Supplementary Information

Functionalization of graphene oxide nanostructures improves photoluminescence and facilitates their use as optical probes in preclinical imaging

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Supplementary Materials and Methods

DLS Characterization of nGO suspensions

Dynamic light scattering and zeta potential: The particle hydrodynamic size and zeta potential were measured in HEPES buffer (25 mM, pH 7.2) by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) measurements, respectively, on a Malvern Zetasizer ZS instrument (Malvern, Worcestershire, UK). The instrument was equipped with a monochromatic red laser beam operating at 632.8 nm and the measurements were performed using the Non-Invasive Back-Scatter (NIBS) technique. The data was analyzed with the Malvern Dispersion Technology Software version 6.20. Z_{ave} values are given as DLS size and/or DLS number.

Transmission electron microscopy

Transmission electron microscopy of nGO nanoparticles (nGO, nGO-Cop, nGO-Cop-FA) were studied by TEM microscopy (JEM-1400 Plus TEM, Jeol Ltd., Japan). The TEM was operated at 80-40 kV acceleration voltage. The images were acquired with an OSIS Quemesa 11 Mpix bottom mounted digital camera. nGO nanoparticles were dispersed in ethanol and then the suspended solution was transferred to electron microscopy grids. The suspension was dried overnight in a desiccator.

Atomic force microscopy

Solutions of nano particles were put onto freshly cleaved mica and dried. AFM imaging was performed with Agilent AFM 5500ILM (Agilent, USA) and surface topography was measured with intermittent contact mode under ambient conditions (T=24-25 degree C, RH=17-34%) using an uncoated rectangular silicon probe (MikroMash, model NSC19/NoAl). Images were recorded with a lateral pixel size of 3.9 nm and a line scan speed of 0.50 Hz. Before measurements of particle sizes, the images were processed with scanning probe image processor software (Gwyddion 2.28) sequentially using four functions in Gwyddion, Level data, Correct lines, Correct horizontal scars, and Remove polynomial background.

In vitro microscopy sample preparation.

In vitro (fixed) cell imaging: HeLa cells were grown in DMEM growth medium (10%FCS, 1% amino acids, 1% penicillin-streptomycin) at 60-70% confluent over coverslips. 20 µg/ml of a) pure nGO, b) nGO-Cop, c) nGO-Cop-FA particles were prepared in 1 ml of cell growth media. The particles were sonicated and vortexed to allow proper de-aggregation of particles, if occurring. Then, the cell media with particles was added to cells growing over coverslips. After incubation for 24h, the media was removed and cells were washed 2X with media and 1X with PBS. Cells were then fixed with 4% PFA for 10 min at RT. After 10 min cells were washed 3X with PBS. 2 µM of Hoechst nuclear dye was added to fixed cells for 10 min to stain the nucleus. While negative control cells were untreated with particles, only 2 µM Hoechst nuclear dye was applied for 10 min after fixation with 4% PFA. Both particle-incubated cells and negative control cells were washed 3X and mounted over glass slides for imaging.

Cancer cells for implantation on CAM Model

MDA-MB-231 breast cancer cell line: The cells were maintained in DMEM, Sigma life sciences, USA (10% FCS, 1% amino acids, 1% penicillin-streptomycin) in a 6 well plate. Cell media was removed and replaced with growth medium containing 10 µg/ml of nGO-Cop-FA pre-sonicated nanoparticles. The cells were incubated with 10 µg/ml nGO-Cop-FA for 24h. 1 million cells were suspended in 10 µl of DMEM (serum free) and mixed with 10 µl of BD Matrigel (BD Biosciences, USA) on ice. Then, the cells were carefully placed in a plastic ring in close proximity to blood vessels. Subsequently, the nGO-Cop-FA incubated

tumors were allowed to develop for the next 5-6 days.

HeLa cervical cancer cell line (FR expression): The cells were maintained in DMEM, Sigma life sciences, USA (10%FCS, 1% amino acids, 1% penicillin-streptomycin) in a 6 well plate. Cell media was removed and replaced with growth medium containing 10 µg/ml nGO-Cop-FA pre-sonicated nanoparticles. Cells were incubated with 10 µg/ml nGO-Cop-FA for 24h. 1 million cells were suspended in 10 µl of DMEM (serum free) and mixed with 10 µl of BD Matrigel (BD Biosciences, USA) on ice. Then, cells were carefully placed in a plastic ring in close proximity to blood vessels. Then, nGO-Cop-FA incubated tumor was allowed to develop for next 5-6days.

A459 lung cancer cell line (FR expression): The cells were maintained in RPMI-1640, Sigma life sciences, USA (10%FCS, 1% amino acids, 1% penicillin-streptomycin) in a 6 well plate. Cell media was removed and replaced with growth medium containing 10 µg/ml nGO-Cop-FA pre-sonicated nanoparticles. Cells were incubated with 10 µg/ml nGO-Cop-FA for 24h. 1 million cells were suspended in 10 µl of RPMI-1640 (serum free) and mixed with 10 µl of BD Matrigel (BD Biosciences, USA) on ice. Then, cells were carefully placed in a plastic ring in close proximity to blood vessels. Then nGO-Cop-FA incubated tumor was allowed to develop for next 5-6days.

In vitro cell imaging

The fixed cell samples of HeLa cells incubated with nGO, nGO-Cop, and nGO-Cop-FA particles and nuclear staining performed with Hoechst dye was imaged using TCS SP5 STED (Leica Microsystems), LASAF software (Leica application suite), PMT and 100X oil objectives. The imaging was performed using sequential scanning option consisting of confocal excitation for nGO particles; while two-photon excitation was used for Hoechst dye. Argon laser excitation at 488nm was used for nGO and Ti-sapphire femtosecond pulse excitation at 740 nm was used for Hoechst nuclear dye. The emission from nGO particles was collected by HyD detectors at 528-630 nm and Hoechst emission was collected in the blue wavelength range.

Excitation dependant emission

The fixed cell samples of HeLa cells incubated with nGO-Cop particles and nuclear staining performed with Hoechst dye was imaged using Leica TCS SP5 STED (Leica Microsystems), LASAF software (Leica application suite), PMT and 100X oil objectives. The imaging was performed using sequential scanning option consisting of confocal excitation for nGO-Cop particles, while two-photon excitation was used for Hoechst dye. Different argon laser excitation at 458 nm, 488 nm, 514 nm, 633 nm was used for nGO-Cop incubated cells. Emission from particles were collected between 470-500 nm for excitation at 458 nm, 528-558 nm for excitation at 488 nm, 558-588 nm for excitation at 488 nm, 584-618 nm for excitation at 514 nm and 649-703 nm for excitation at 633 nm. The average of emission wavelength (i.e. (470+500)/2= 485 nm) was calculated in terms of RGB values and corresponding real RGB value were allotted to cells. Ti-sapphire femtosecond pulse excitation at 740 nm was used for Hoechst nuclear dye. The Hoechst emission was constantly collected in the blue wavelength range.

Supplementary Figures

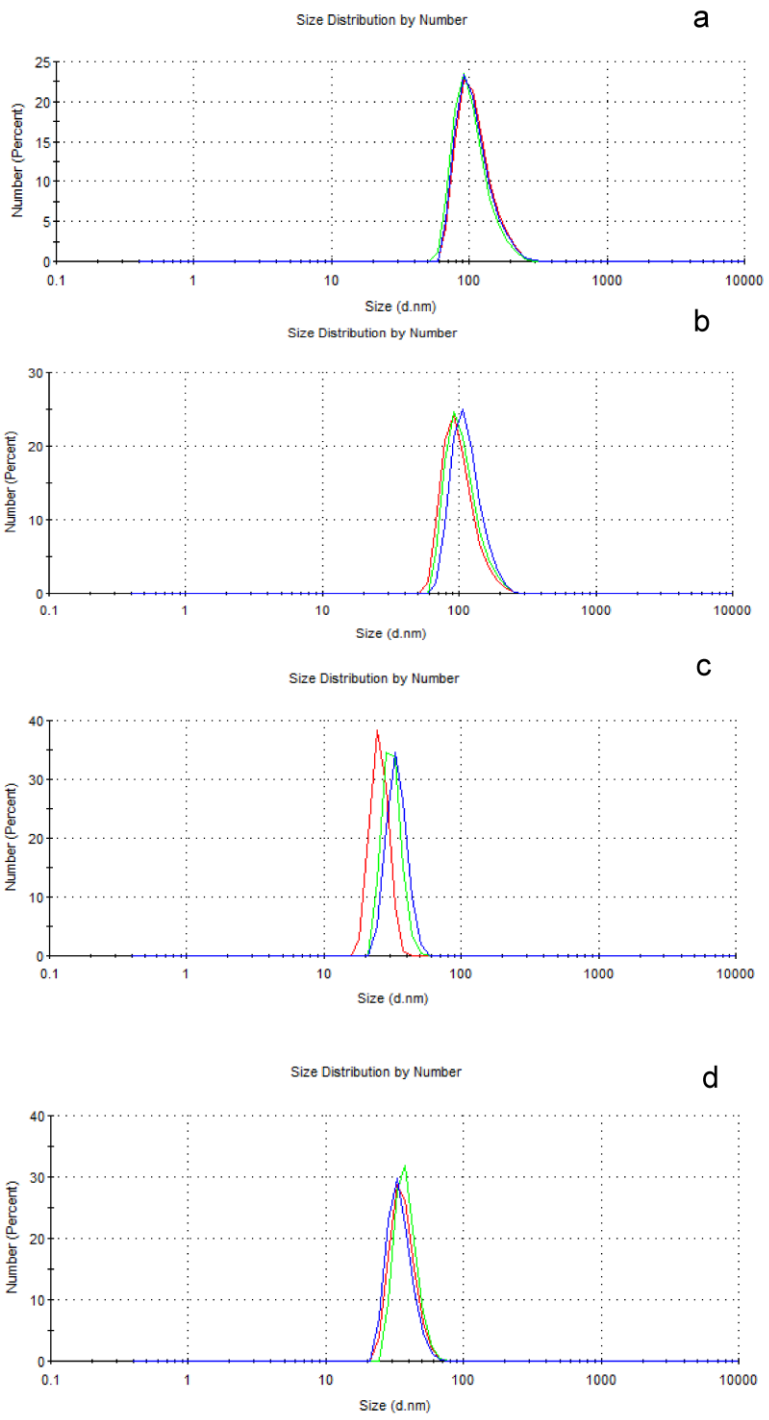

Figure S1. Dynamic light scattering was used for determining the hydrodynamic size of a) nGO ,b) nGO-cop 10 wt-%, c) nGO-cop 20 wt-% and d) nGO-cop 30 wt-%. The DLS measurements were carried out in HEPES buffer (25 mM, pH 7.2).

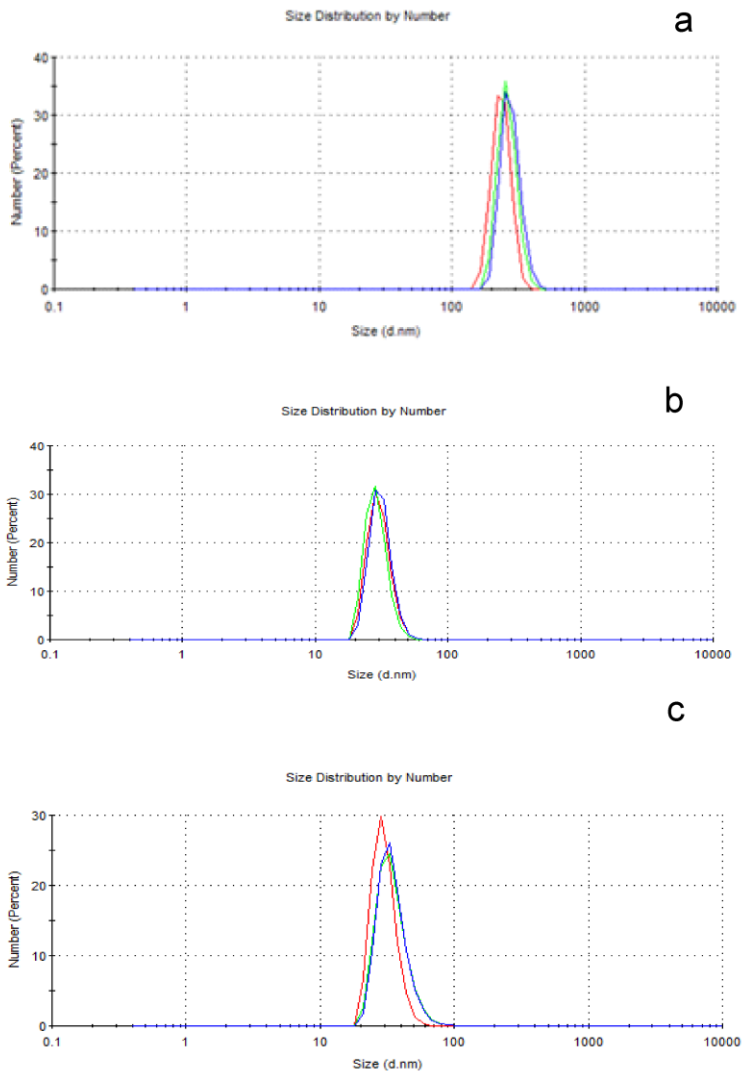

Figure S2. Dynamic light scattering was used for determining the hydrodynamic size of a) nGO-cop@FA 10 wt-%, b) nGO-cop@FA 30 wt-% and c) nGO-cop@FA 50 wt-%. The DLS measurements were carried out in HEPES buffer (25 mM, pH 7.2).

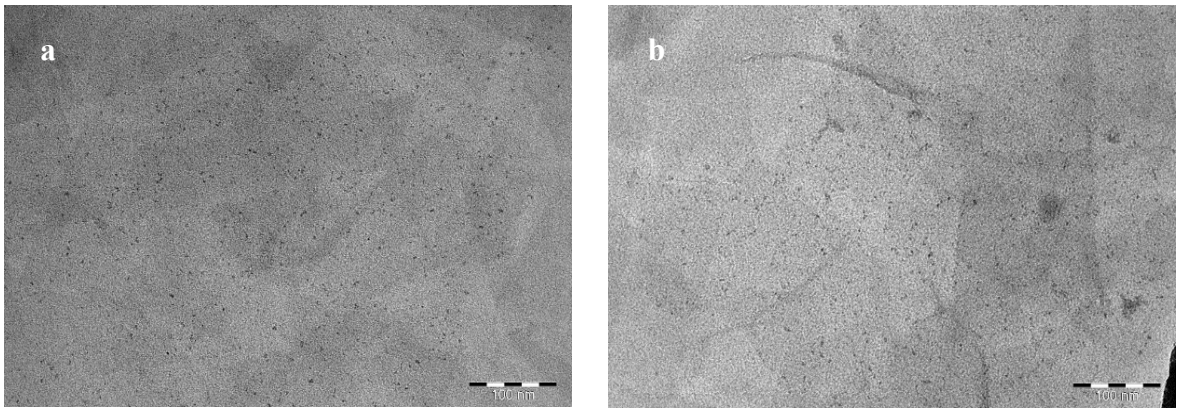

Figure S3. Transmission electron microscopy of nGO nanoparticles (a) nGO-cop and b) nGO-cop-FA) were studied by TEM microscopy (JEM-1400 Plus TEM, Jeol Ltd., Japan). The TEM was operated at 40 kV (low voltage) acceleration voltage.

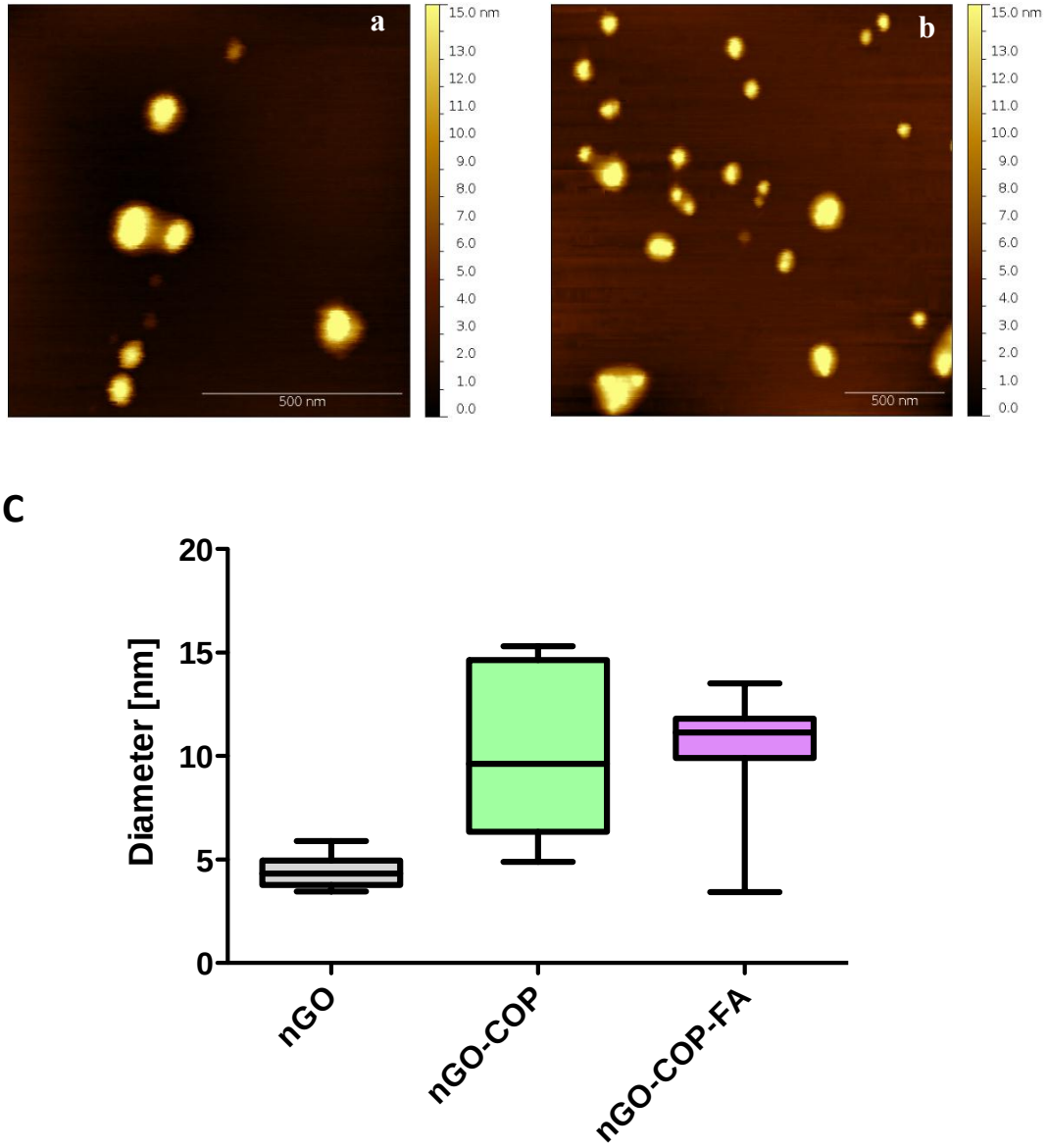

Figure S4. Atomic force microscopy characterization of nGO nanoparticles (a) nGO-cop and (b) nGO-cop-FA in dried specimens to exclude the effect of aggregation and hydrodynamic effects. The samples were spin-coated and dried on freshly cleaved mica after stringent dispersion. Comparison of line profile heights nGO 4.3 nm; COP 9.6 nm; COP-FA 11.1 nm (c) was performed on smallest found entities (N=10) in order to exclude aggregates from the analysis. Boxes show median values and 95% confidence intervals and whiskers minimum and maximum values.

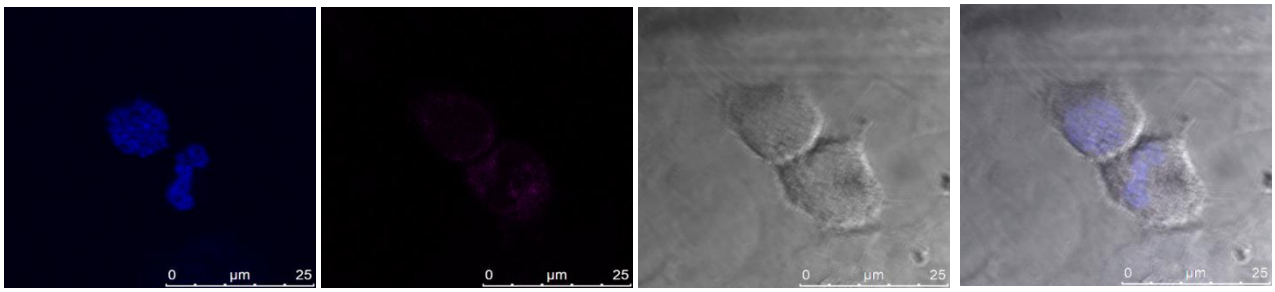

Figure S5. Control HeLa cells at the division stage.

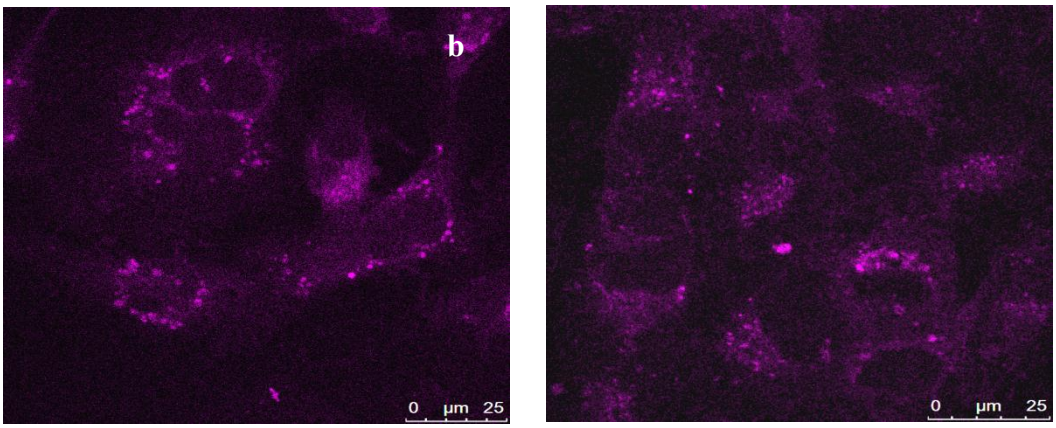

Figure S6. PL images from fixed a) MDA-MB-231 and b) A549 cells incubated with a 10 µg/ml nGO-Cop-FA for 7h. Confocal imaging: Argon laser Ex.488nm, Em.520-580nm.

Movie S7. *In vivo* detection of HeLa cancer cells labeled with nGO-Cop-FA and after one week of implantation over chicken CAM (separate file)