

Supporting Information

Versatile Multicolor Nanodiamond Probes for Intracellular Imaging and Targeted Labeling

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Experimental Section

Fabrication of SiV FNDs: The growth of dispersed FNDs containing SiV color centers was performed using a microwave plasma CVD reactor, with a hydrogen/methane ratio of 50:1 at 50 Torr, a microwave power of 1500 W, equipped with a cooling stage, from 4–6 nm detonation FNDs seeds on a silicon wafer. The unwanted silicon substrates were dissolved in 30% potassium hydroxide (KOH) solution at 89°C for several hours. The detached polycrystalline diamond thin films were separated from the KOH solution using a corning filtration system with a 0.22 µm polyethersulfone membrane. The separated diamond film fragments were subsequently dissolved in dimethyl sulphoxide (DMSO).

To fabricate individual ND particles, diamond thin film was disintegrated into nanoparticles by Bead-Assisted Sonication Disintegration (BASD) process. Specifically, 10 mg of zirconia beads were added to the diamond film solution and subjected to sonication (probe sonicator) for 2 hours with a 2/1 second on/off pulse. The zirconia and DMSO were removed through centrifugation and washed with water. To remove residual zirconia fragments and graphite, the diamond solution then underwent acid reflux with a 1:1 solution of nitric and sulphuric acid in an oil bath at 83°C for 24 hours. The acid solution was removed by centrifugation for 30 minutes and replaced with water. The size of the particles was measured using dynamic light scattering (DLS) and were shown to be $\sim 141.01 \pm 40.12$ nm in diameter with a zeta potential of -17.40 ± 3.60 mV indicating that the FNDs containing SiV color centers are relatively small and dispersed in solution. To unambiguously show that the particles are diamond, Raman spectroscopy equipped with 633 nm laser was employed. To prove that the diamond particles host bright SiV color centers, the solution was characterized using a home built scanning Hunbury Brown and Twiss interferometer using a 532 nm excitation source through a high numerical aperture (NA = 0.9) objective at room temperature.

Surface modification of NV containing FNDs: To create the NV FNDs-TAT complex 300 µL of 1 mg/mL solution of FNDs containing NV color centers was mixed with 5 µL of 10 mg/mL 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 50 µL of 10 mg/mL N-Hydroxysuccinimide (NHS). After sonication 200 µL of 2.5 mg/mL of TAT peptides in HEPES buffer was added then sonicated for an additional 2 hours to ensure complete surface coverage.

Growth of cells: Chinese Hamster Ovary (CHO-K1) cells are grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Life Technologies) at 37°C + 5% CO₂ for a couple of days to reach ~ 80% confluency. To lift off cell from the plastic container, ~2 mL of 5 mM Ethylenediaminetetraacetic acid (EDTA) in serum-free media, (DMEM/F12) to cover the cells for 2-5 minutes at room temperature. Lifted cells were diluted with ~10 mL of 5% Fetal Bovine Serum (FBS) in DMEM/F12, transferred to a 15 mL falcon tube, and centrifuged at 1200 rpm for 4 minutes. 1/10 of the cells were split and transferred into a new flask. The cells are then grown for the desired time (1-3 days) at 37°C + 5% CO₂.

Macrophages: The monoblast-like human histiocytic lymphoma cell line U937 can be induced by phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) at concentrations of 8-16 ng/ml to undergo differentiation to macrophages. U937 cells were cultured in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum at 37°C + 5% CO₂.

Cell viability of FNDs in cells: To assess cell viability of cell with FNDs, NV and SiV containing FNDs were incubated with two types of cells at concentrations ranging from 5 µg/mL - 100 µg/mL.

CHO-K1 cells were grown in DMEM-F12 Glutamax cell culture medium, supplemented with 5% fetal bovine serum (FBS), and the resultant cell suspension (~1x10⁵ cells/mL) was dispersed into 96-well plates and incubated overnight to allow for cell adherence. The required concentration of FNDs were added dropwise into 1 mL of cell media and incubated with the CHO-K1 cells at 37°C for 3 hours. Cells without FNDs were grown under identical conditions were used as controls.

The monoblast-like human histiocytic lymphoma cell line U937 can be induced by phorbol 12-myristate 13-acetate (PMA) at concentrations of 8-16 ng/ml to undergo differentiation to macrophages. U937 cells were cultured in DMEM/F12 supplemented with 10% heat-inactivated FBS at 37°C + 5% CO₂. To assess cell viability, 0.2 X 10⁶ U937 cells were seeded into 24 well plates (BD Biosciences, USA) such that they were 70-80% confluent at the time of assay. 15 ng/ml PMA solution was added to each well and cells were incubated for 48 hours at 37°C + 5% CO₂. The cells were then subsequently washed with PBS and replaced with fresh media and allowed to grow for another 4 hours, after which NV and SiV containing FNDs were incubated with macrophage cells at concentrations ranging from 5 µg/mL - 100 µg/mL. Macrophages were incubated with the FNDs at 37°C + 5% CO₂ for 3 hours. Cells alone, grown under identical conditions were used as controls.

200 µL of 1 mg/ml of TAT peptide dispersed in 800 µL of DMEM/F12 media served as additional control for cell viability with only TAT peptide.

Cell viability was determined by the AlamarBlue (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) assay (Life Technologies, Australia) according to the manufacturer instructions. The absorbance of cells with AlamarBlue reagent and various diamond concentrations in addition to controls was measured at 600 nm using a microplate reader. **Figure S2** shows the percentage (%) of cell viability following exposure to varying FNDs concentrations after 4 hours of incubation. Additionally, to assess the effect of FNDs over a sustained exposure time, the samples were measured after 24 hours (Figure S2 b). The cell viability percentage was determined by the absorbance of the FNDs in cells divided by the absorbance of the control. Error bar represents standard deviation between at least three independent measured samples.

Immunofluorescence staining: After cells have reached confluency, cells were split as described previously. ~0.5 x 10⁶ cells with cell media were transferred to a 35 mm Fluorodish cell culture dish purchased from World Precision Instruments (WPI). The cells were incubated overnight at 37°C + 5% CO₂ to reach optimal confluency and to allow the cells to adhere to the surface of the imaging dish. 60 µL of the 50 µg/mL of FNDs was transferred to the imaging dish and incubated for a desired time to ensure uptake of the FNDs. The media was removed and replaced with 1 mL of 4% paraformaldehyde in PBS and incubated at room temperature for 15 minutes. The sample is then washed thrice with 1 mL of PBS for 2-5 minutes. To allow for fluorescence labelling of cell interior, 1 mL of permeabilization solution (0.5% Triton X-100) was added and incubated at room temperature for 15 minutes. The sample was subsequently

washed and replaced with 1 mL of blocking solution (3% BSA in DPBS) for one hour to reduce the amount of nonspecific binding in the sample, replaced with 1 mL of specific primary antibodies (LAMP-1 or RAB7, Life Technologies) diluted 1:1000 in a blocking solution and incubated for one hour at room temperature. The sample was washed and replaced with 1 mL of Alexa Fluor 488-labeled secondary antibody diluted 1:1000 in PBS and incubated for one hour in the dark at room temperature. The sample is washed and 2 drops of NucBlue (nucleus staining reagent) was added prior to imaging. Fixed and stained cells were imaged using an A1 Nikon confocal scanning laser microscope at room temperature equipped with 405 nm, 488 nm and 561 nm excitation laser sources. The samples were then excited by a Coherent OBIS 561 nm continuous wave excitation source, the signal was then passed through a photo-multiplier tube/spectrometer with a collection range from ~570 nm to 740 nm for confocal microscopy cellular studies.

Co-localization comparison: The various imaging channels for the nucleus, fluorescent biomarker and FNDs (blue, green and red) were quantified using Pearson's Correlation coefficient, Costes, Manders coefficient analysis using Fiji (ImageJ) software. The confidence of co-localization between two color channels, such as the green LAMP1 or RAB7 stained channel and NIR FND channel, increases as the Pearson's coefficient R tends towards 1. An R value of 0 is equivalent to random noise. The Manders Coefficient M1/M2 is a secondary check on the validity of the test and tends towards 1; it describes the degree of overlapping between the red and green pixels of our particles and stain respectively. Similarly, the Costes P-value and Li's intensity correction quotient (ICQ) values are separate validity checks and tend towards 95% and 0.5, respectively, when there is co-localization between two images.

The Pearson's coefficient calculates the degree of overlap between the pixels in the red (FNDs) and green (fluorescent biomarker) color channels using the equation¹:

$$r = \frac{\Sigma(\text{red intensity} - \text{average red}) \times (\text{green intensity} - \text{average green})}{\sqrt{\Sigma(\text{red intensity} - \text{average red})^2 \times \Sigma(\text{green intensity} - \text{average green})^2}}$$

According to the Pearson correlation, perfect correlation equals to 1, perfect negative correlation equals to -1 and 0 denotes the absence of a relationship.

The Manders coefficient calculates according to the following equation:

$$M_R = \frac{\sum_i R_{i,coloc}}{\sum_i R_{i,total}}, M_G = \frac{\sum_i G_{i,coloc}}{\sum_i G_{i,total}}$$

where M_R is the ratio of the summed intensities of pixels from the green image for which the intensity in the red channel is above zero to the total intensity in the green channel and M_G is conversely for red. $R_{i,coloc}$ is the intensity of a particular pixel and $R_{i,total}$ is the total intensity of the channel, similarly, G_i , for the green channel.

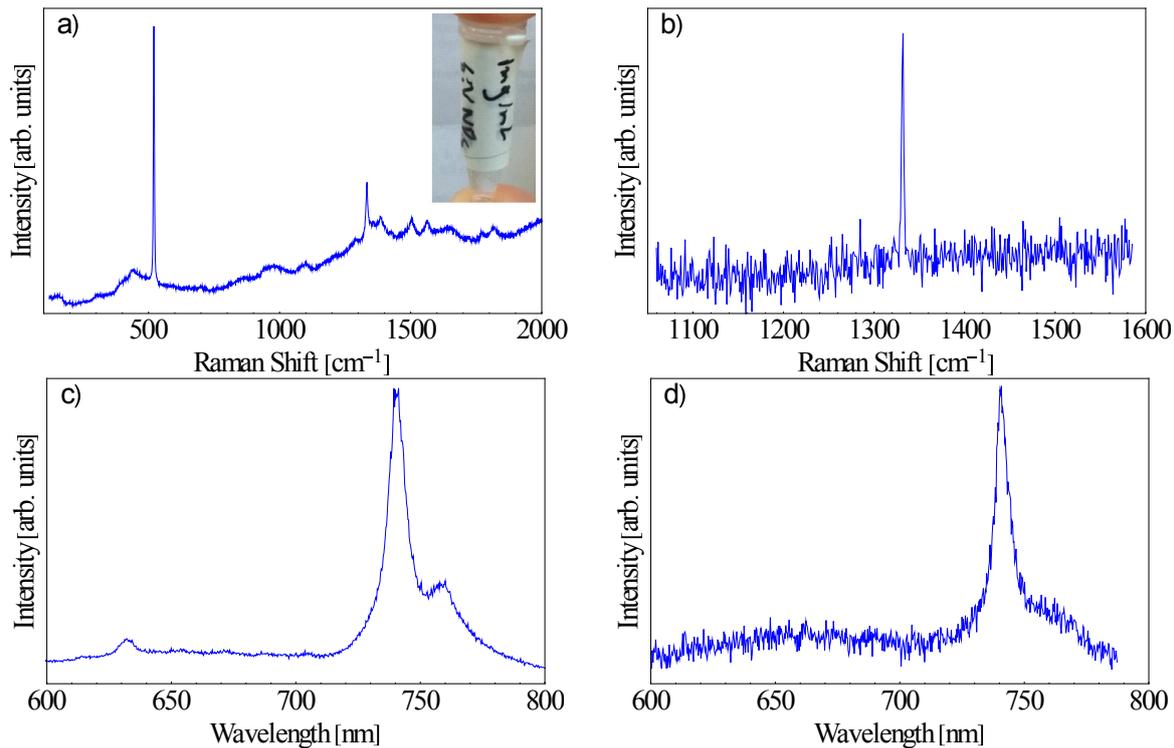


Figure S1. Characterization of fabricated diamond films and particles. (a) Raman spectra of CVD grown diamond particles with a 633 HeNe laser. It shows the characteristic silicon and diamond peak at 520 cm^{-1} and 1333 cm^{-1} respectively. Inset: Photo of the processed CVD grown particles in aqueous solution. (b) Raman spectra of processed FNDs after purification, exhibiting the Raman diamond peak at 1333 cm^{-1} . (c) Photoluminescence spectra of CVD grown diamond particles at RT using a 532 nm excitation source, it shows the characteristic SiV emission at 738 nm. (d) Photoluminescence spectra of processed FNDs showing that they still host bright SiV color centers.

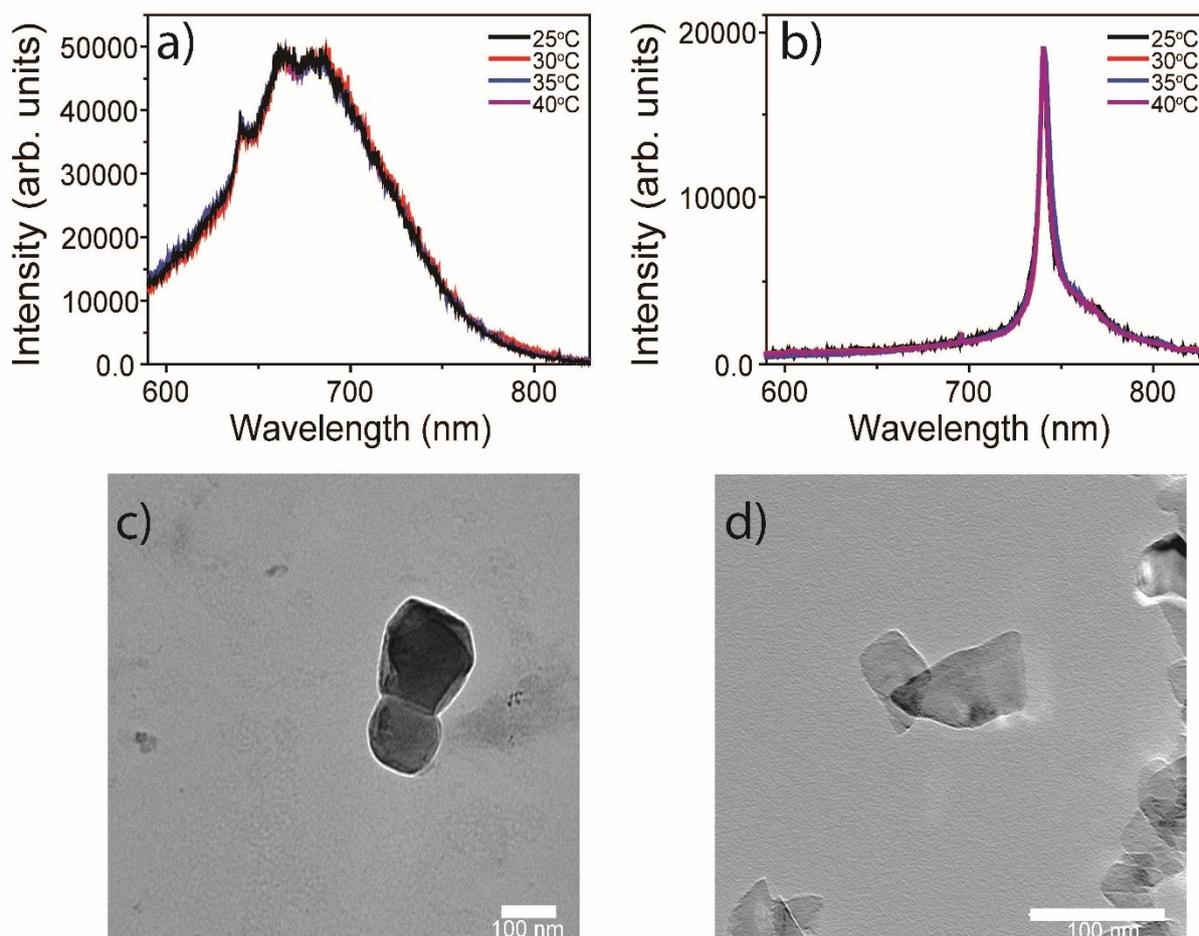


Figure S2. Characterization of fabricated diamond particles. Temperature dependency of FNDs containing either (a) NV or (b) SiV fluorescent defects with a 532 nm laser. Spectra were collected at 25°C, 30°C, 35°C and 40°C. The spectra show the zero phonon line to be consistent as temperature increases. Transmission Electron Microscopy images of (c) NV and (d) SiV containing FNDs.

Table S1. Characterization of various FNDs samples through DLS using a 633 nm HeNe laser source. Standard deviation was calculated based on measurements of at least three different samples.

| Sample | Size distribution by Number (nm) | Size distribution by Volume (nm) | Zeta Potential (mV) |
|---------------|----------------------------------|----------------------------------|---------------------|
| NV FNDs | 44.8 ± 13.7 | 60.9 ± 25.8 | - 45.4 ± 21.2 |
| NV FNDs – TAT | 68.6 ± 22.0 | 99.3 ± 59.7 | + 32.8 ± 5.6 |
| SiV FNDs | 141.1 ± 49.4 | 153.1 ± 39.3 | -17.4 ± 3.7 |

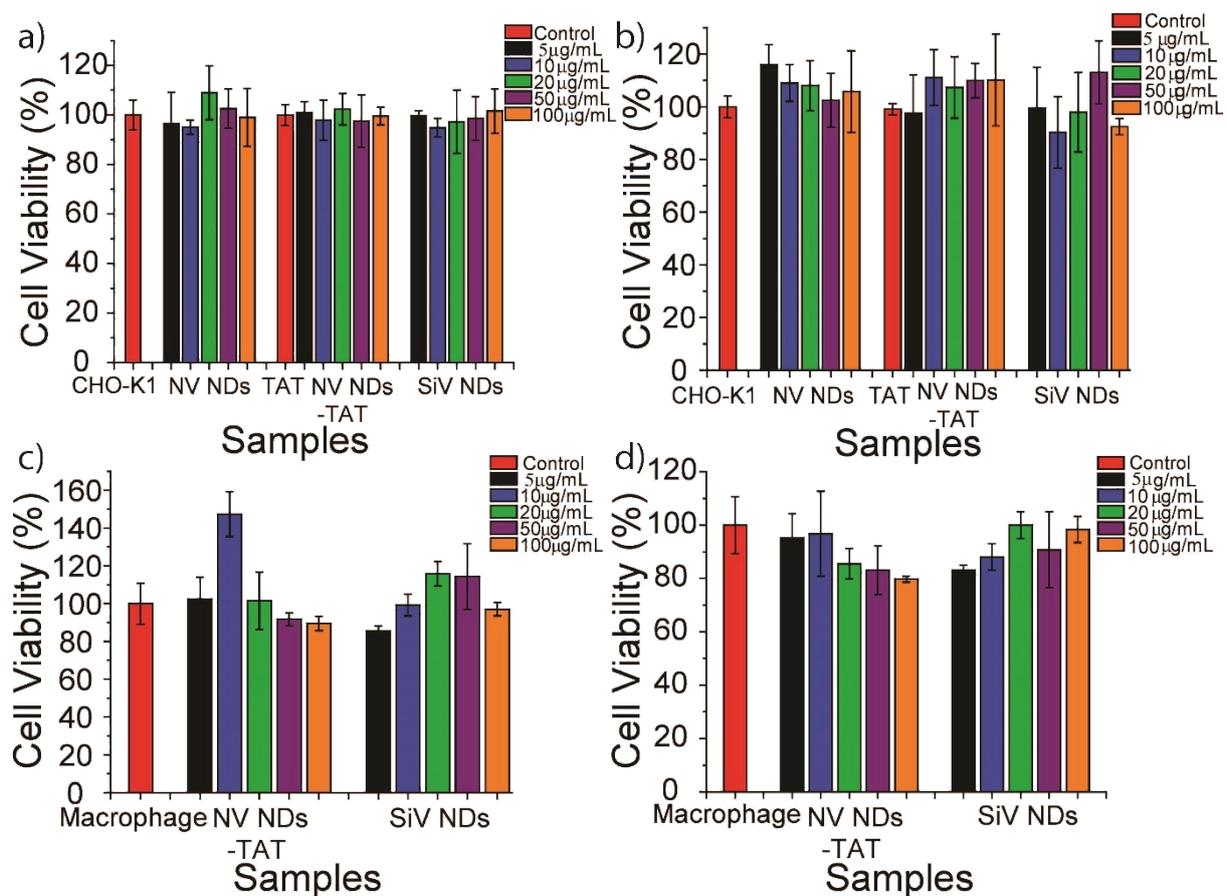


Figure S3. A cell viability assay for FNDs samples of varying concentrations (5 µg/mL – 100 µg/mL) in CHO-K1 and macrophage cells with a controls after (a, c) 4 and (b, d) 24 hours of incubation at 37°C. The small fluctuations show that the FNDs are non-toxic to the CHO-K1 cells. Error bar represents standard deviation of at least three different samples (SD, n=3).

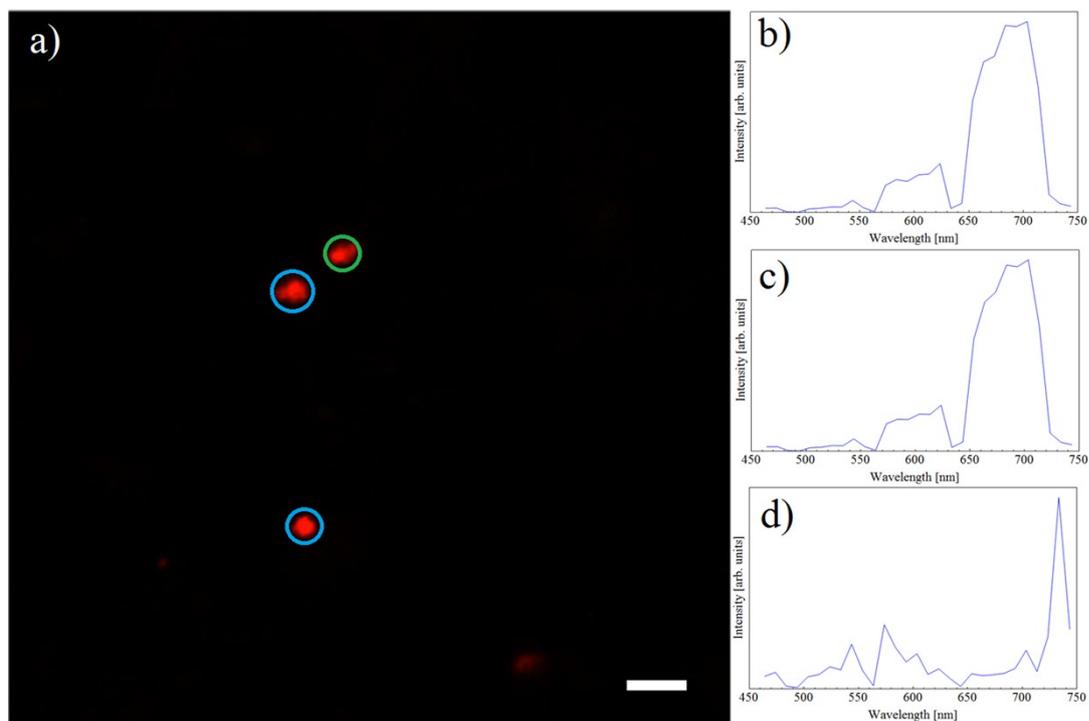


Figure S4. Confocal laser scanning microscopy of fixed CHO-K1 cells. Cells containing FNDs hosting (a) both SiV and NV-TAT were imaged using 561 nm excitation laser and emission is collected by a spectrometer. The NV (blue circles) and SiV (green circles) color centers are labelled for clarity. Scale bar is 10 μm . Spectra were obtained from the circled FNDs and exhibited a strong (b, c) NV and (d) SiV emission.

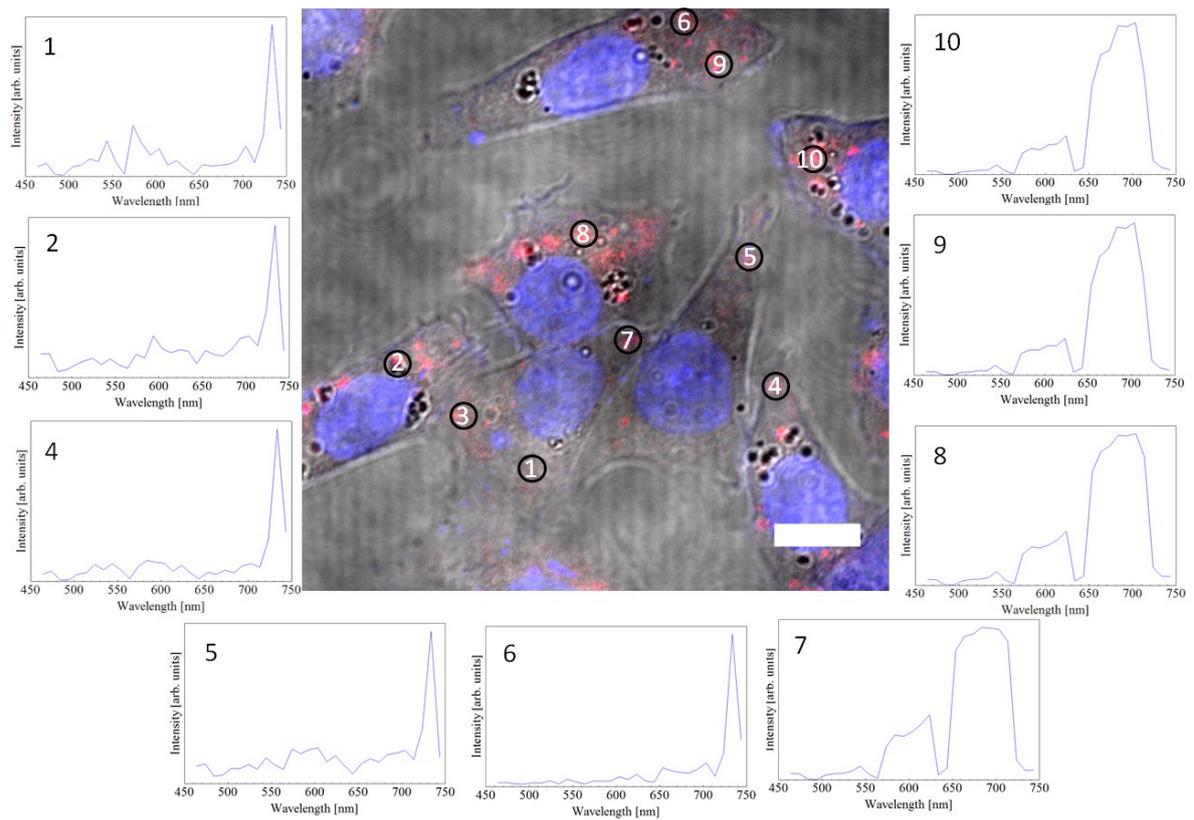


Figure S5. Bright field image merged with fluorescence confocal laser scanning microscopy imaged of fixed CHO-K1 cells. Cells containing FNDs hosting both SiV and NV-TAT were imaged using confocal microscopy equipped with a 561 nm excitation laser and emission collected by a spectrometer. Randomly selected 10 spots were characterized to reveal their spectral signatures. Spectra were obtained from the circled FNDs number 1,2,4,5,6 represent SiV NDs spectral emission, while spectra from spots number 7,8,9,10 are originated from NV NDs emission. Scale bar is 10 μ m.

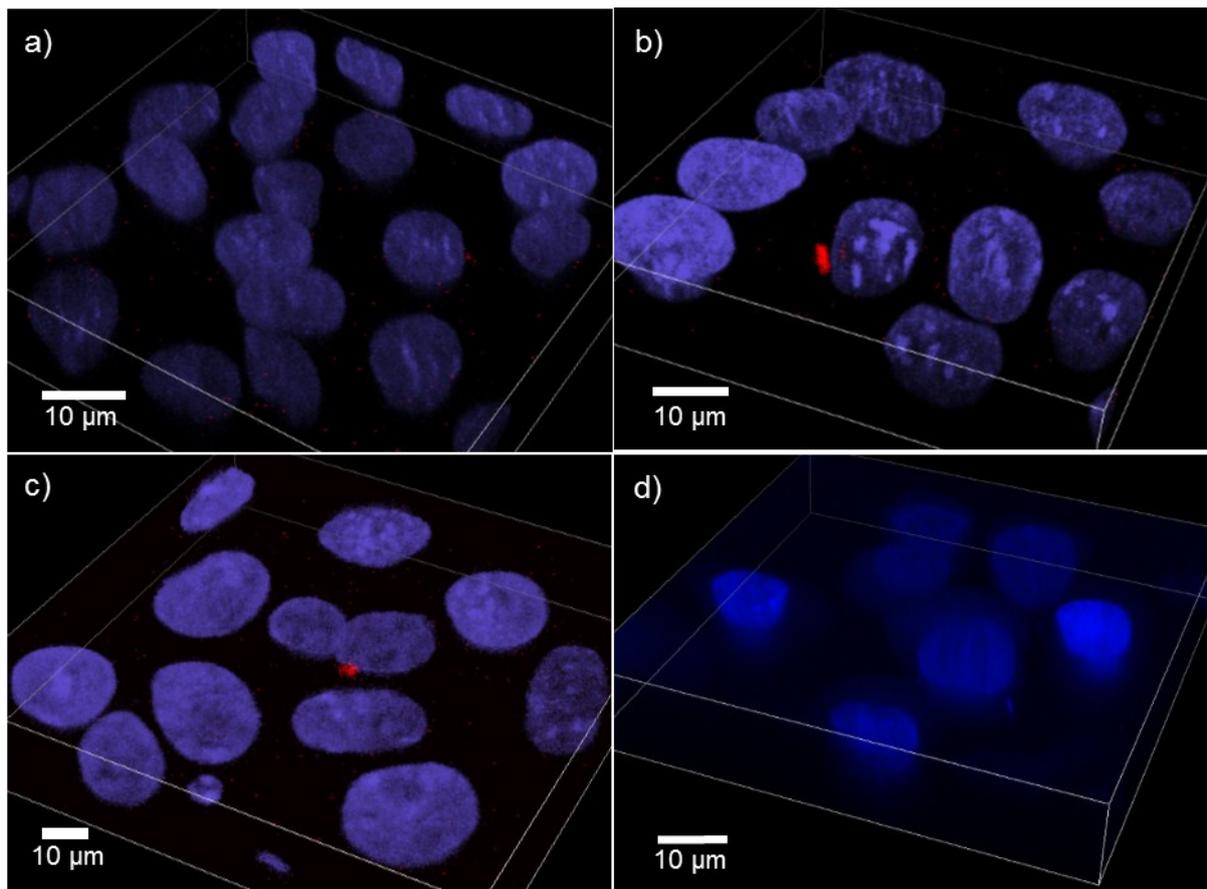


Figure S6. 3D reconstruction of confocal raster scan of CHO-K1 cells hosting (a) SiV (b) NV (c) both SiV and NV-TAT containing FNDs and (d) control cells. 405 nm laser excitation shows the NucBlue stained nucleus of the cells, while 561 nm laser excitation was used to maximize the collected signal from the FNDs particles. Fluorescence from SiV FNDs is dispersed throughout the cell except nucleus, while fluorescence from NV-TAT FNDs is clearly segregated in a perinuclear area of cells.

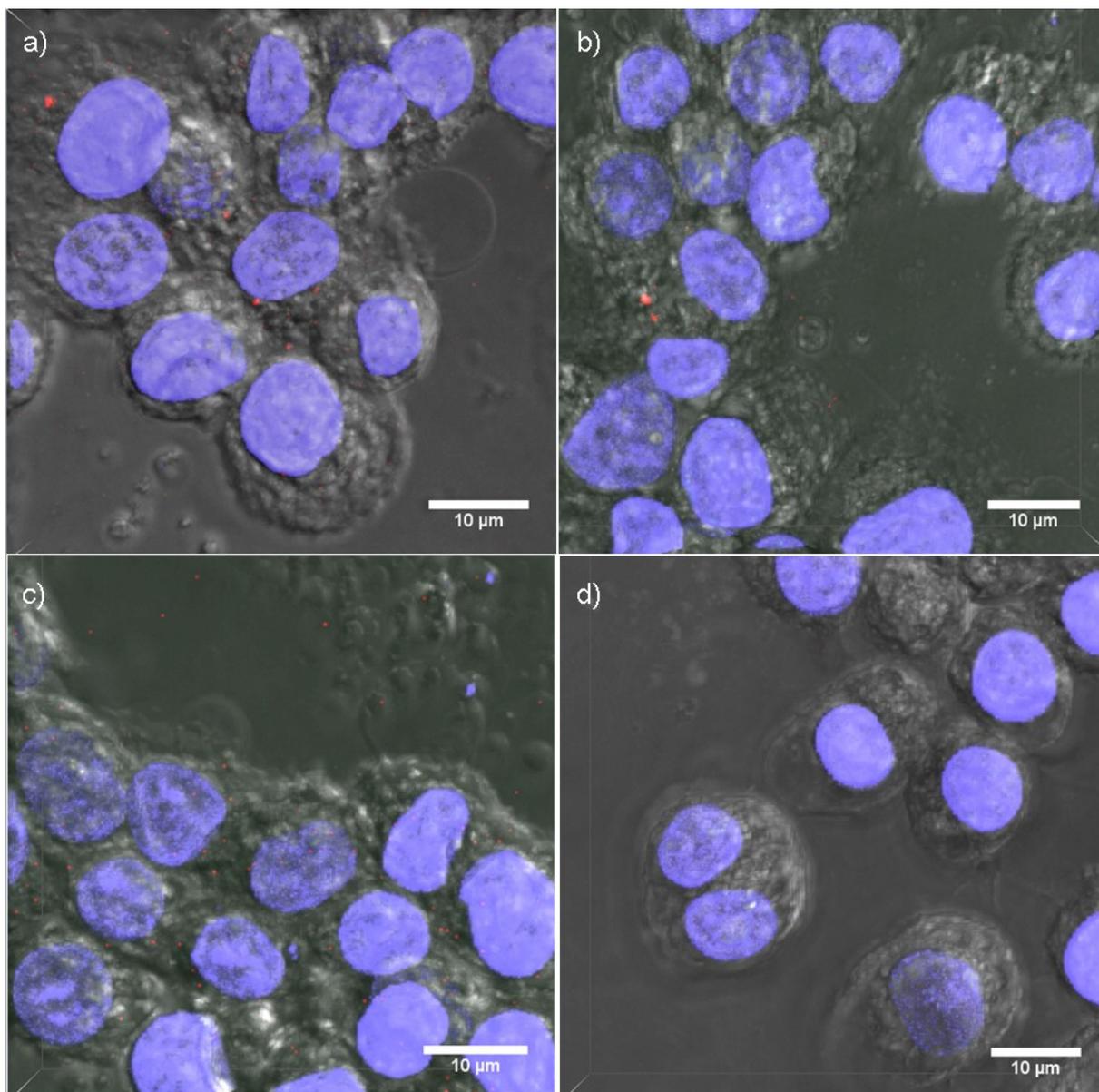


Figure S7. 3D reconstruction of confocal raster scan of U937 cells induced to macrophages hosting (a) SiV (b) NV (c) both SiV and NV-TAT containing FNDs and (d) control cells. 405 nm laser excitation shows the NucBlue stained nucleus of the cells, while 561 nm laser excitation was used to maximize the collected signal from the FNDs particles. Fluorescence from both FNDs is dispersed throughout the cell except nucleus.

References:

1. Adler, J. and Parmryd, I. (2010), Quantifying colocalization by correlation: The Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry*, 77A: 733–742.