Supporting Material to "Nanoparticles Microinjection and Raman spectroscopy as tools for nanotoxicology studies"

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One fluorescence microscopy facility, Nikon Eclipse TE-2000U, has been equipped with a cell microinjection system, InjectMan-NI2 micromanipulator combined with Femtojet microinjector both from Eppendorf (Fig.S1). The micromanipulator allows for movements with a spatial resolution below the μm range (that is more than enough for cells experiments), while the smallest microinjected volumes are in the femtoliter range.

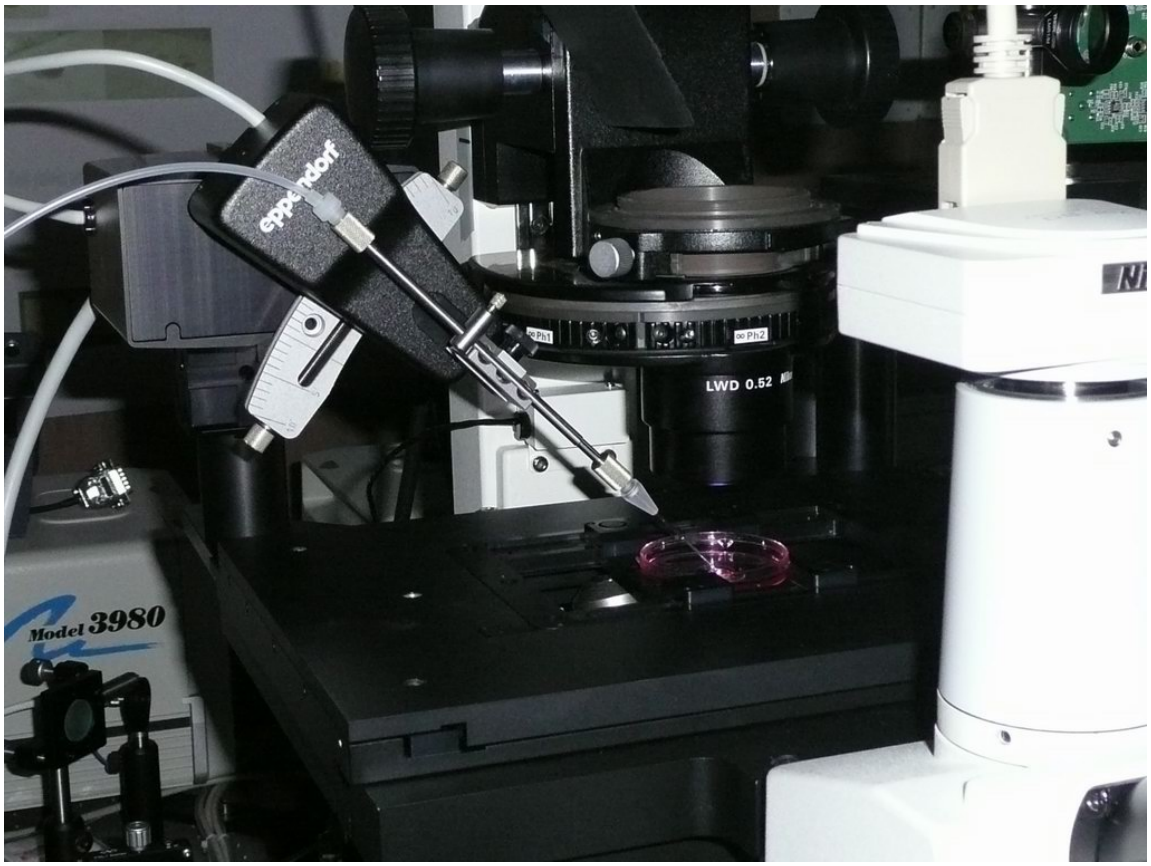

Figure S1: *(color on-line) Eppendorf microinjection setup mounted on Nikon Eclipse TE-2000U.*

Several experiments have been carried out to establish a reproducible and reliable protocol for the single cell/few NPs interaction. The microinjection tips used in the experiments are pre-pulled glass pipettes (purchased from World Precision Instruments, WPI) with an external diameter tip of 1.0μm. Finally, cells are microinjected in the cytoplasm with a pressure ranging between 110 and 140hPa while the injection time is kept constant at 0.7sec. Environmental scanning electron microscopy (ESEM) confirmed the presence of confined amounts of NPs inside the injected cells, while no evidence of NPs was found in the surrounding cells (Fig.S2). In order to make evident the nanoparticles inside the cells during ESEM measurements, the NPs amounts microinjected in the cells are much larger than those used in the experiments for Raman spectroscopy. For this reason in Fig.S2 large NPs clusters

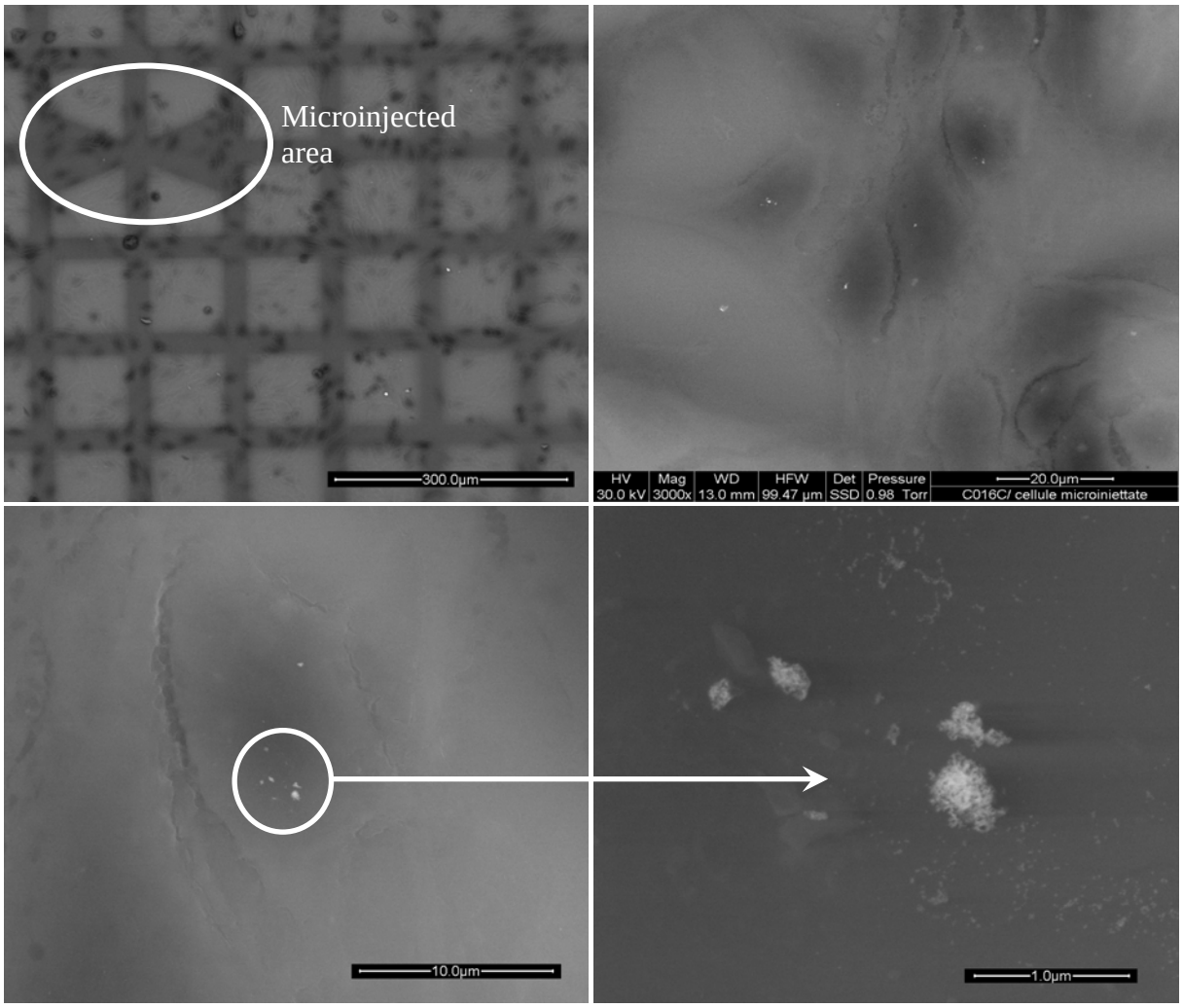

Figure S2: *ESEM pictures of cells microinjected with nanoparticles. Top row on the left, an overview picture of the metal reference grid fabricated on the Petri-dishes for ESEM analysis; in the picture the microinjected area is indicated, easily identified through the two triangular shapes. Top row on the right, group of cells from the microinjected area with NPs observable in the cells themselves. Bottom row, detailed pictures of one microinjected cell (on the left) and the clusters of nanoparticles found inside the cell (on the right). The ESEM analysis confirmed that nanoparticles are found only in the microinjected cells and not in the surrounding ones.*

are observable inside the cells. From the ESEM pictures we could estimate the volume of the NPs clusters and, since the single NP volume is known, we could estimate the total number of injected NPs. In the case of Fig.S2 we have around 30000NPs for each injection. Keeping constant the microinjection parameters and diluting the NPs solutions, we could adjust the microinjection experiments so that to deliver 10-50NPs to each cell.

Besides the effective presence of NPs in the cells, another important point to be addressed for the microinjection protocol is the cellular damage that could be caused by the technique itself (due to the mechanical stress of the cells). For this purpose a GFP-plasmid transfection experiment has been carried out by means of microinjection. More in details, Hela cells are microinjected with a GFP-plasmid solution and after an incubation time of 24hrs the GFP expression is detected through fluorescence microscopy. Among the microinjected cells, only the healthy ones can carry out the protein synthesis and produce the GFP protein (Fig. S3).

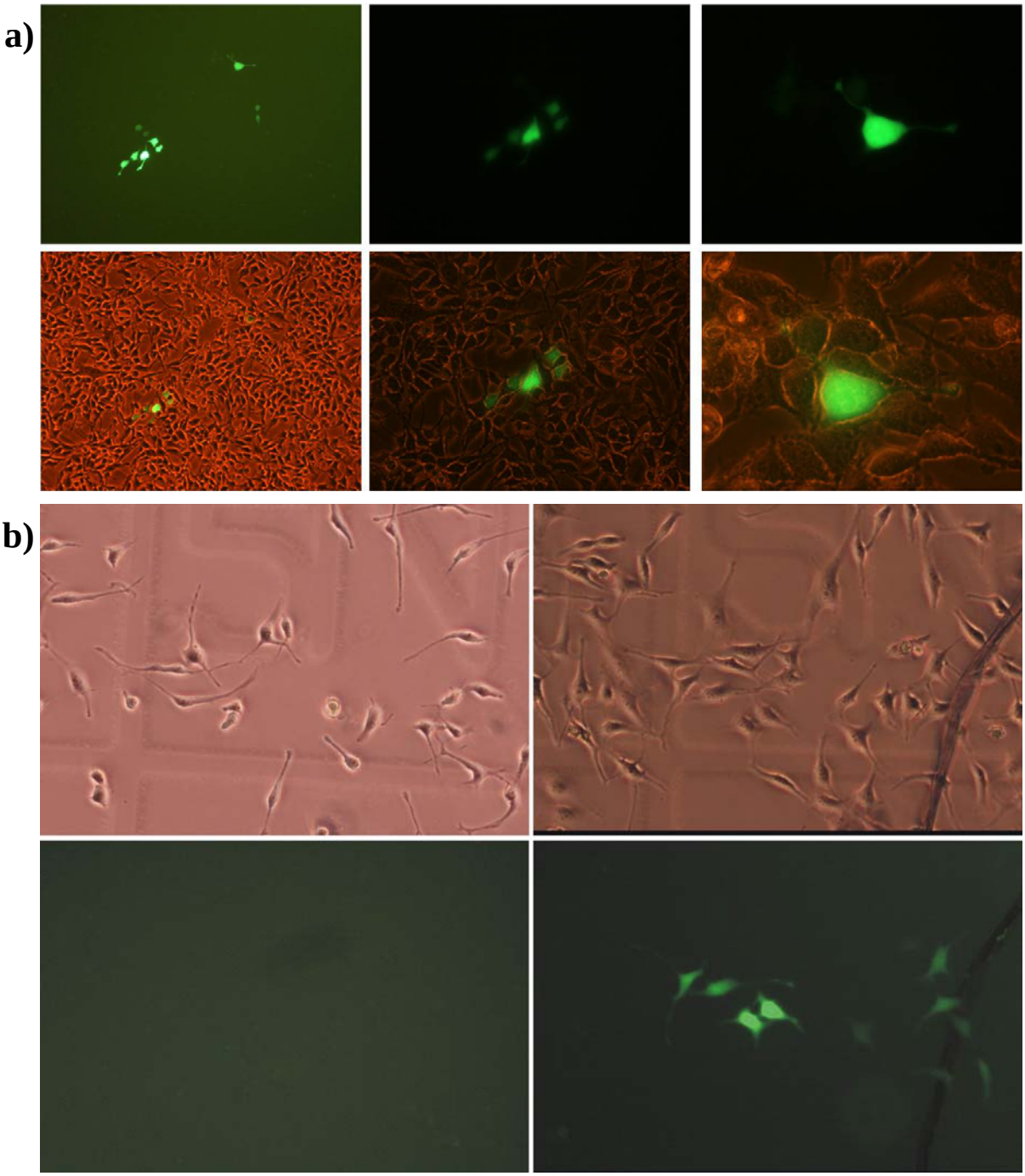

Figure S3: *(color on-line) GFP-plasmid transfection experiment of Hela cells by means of microinjection technique. In panel a), fluorescence (top row) and fluorescence plus white-light (bottom row) microscopy images of injected cells 24hrs of incubation after the microinjection are reported. In panel b), the white light (top row) and the fluorescence (bottom row) microscopy images soon after the microinjection (on the left) and 24hrs of incubation later (on the right) are shown. During the incubation time, only microinjected cells in a healthy status can carry out the GFP synthesis, thus becoming green fluorescent.*

As a consequence, the fluorescence analysis provides a direct picture of the successfully microinjected cells. According to this experiment, an average of 75% of successfully injected cells is achieved with the established protocol.

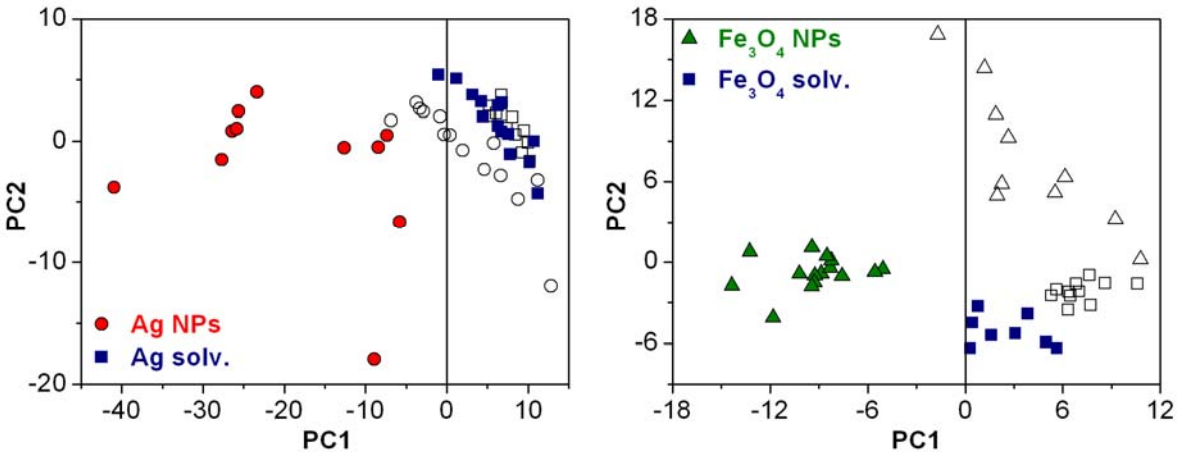

Figure S4: *PCA analysis performed over the spectra coming from the cells treated with Ag NPs and Ag-solvent (on the left), and coming from the cells treated with Fe3O4 NPs and Fe3O4-solvent (on the right), along with the corresponding control cells. On the left, full circles and full squares correspond respectively to Ag NPs and Ag-solvent treated cells; on the right, full triangles and full squares correspond to Fe3O4 NPs and Fe3O4-solvent treated cells. In both graphs, the open symbols refer to control cells. The scores corresponding to cells treated with solvents are located close to the scores coming from control cells, thus confirming that no significant differences can be found between control cells and solvents-treated cells.*

In order to prove that the effects observed in the case of the cells microinjected with the nanoparticles (see the "Results and Discussion" section) are not due to the microinjection technique itself, similar experiments are carried out microinjecting the Hela cells only with the NPs solvents. The Raman spectra measured for the solvents injected cells are shown in the "Results and Discussion" section (Fig. 5), along with the spectral differences. The spectral differences do not show any significant effect due to the solvents and/or to the mechanical stress of the microinjection technique. Also PCA analysis performed over the spectra from solvents injected cells, nanoparticles injected cells and corresponding control cells does not highlight any influence due to solvents and/or microinjection. As reported in Fig. S4, the score plots show that the solvents treated cells are very close to the control cells, while the nanoparticles treated cells are separated from both the solvents treated cells and the control ones. This is a further confirmation that solvents and microinjection technique itself do not affect significantly the cells activities, and that the effects observed in the case of NPs microinjection are only due to the NPs themselves.

Dr. A. Gatti and Dr. G. Sighinolfi from CNISM at the University of "Modena and Reggio Emilia" are gratefully acknowledged for the ESEM pictures.