

# 1 Supplementary information

## 2 Hyperspectral enhanced dark field microscopy for 3 single and collective nanoparticle characterization in 4 biological environments

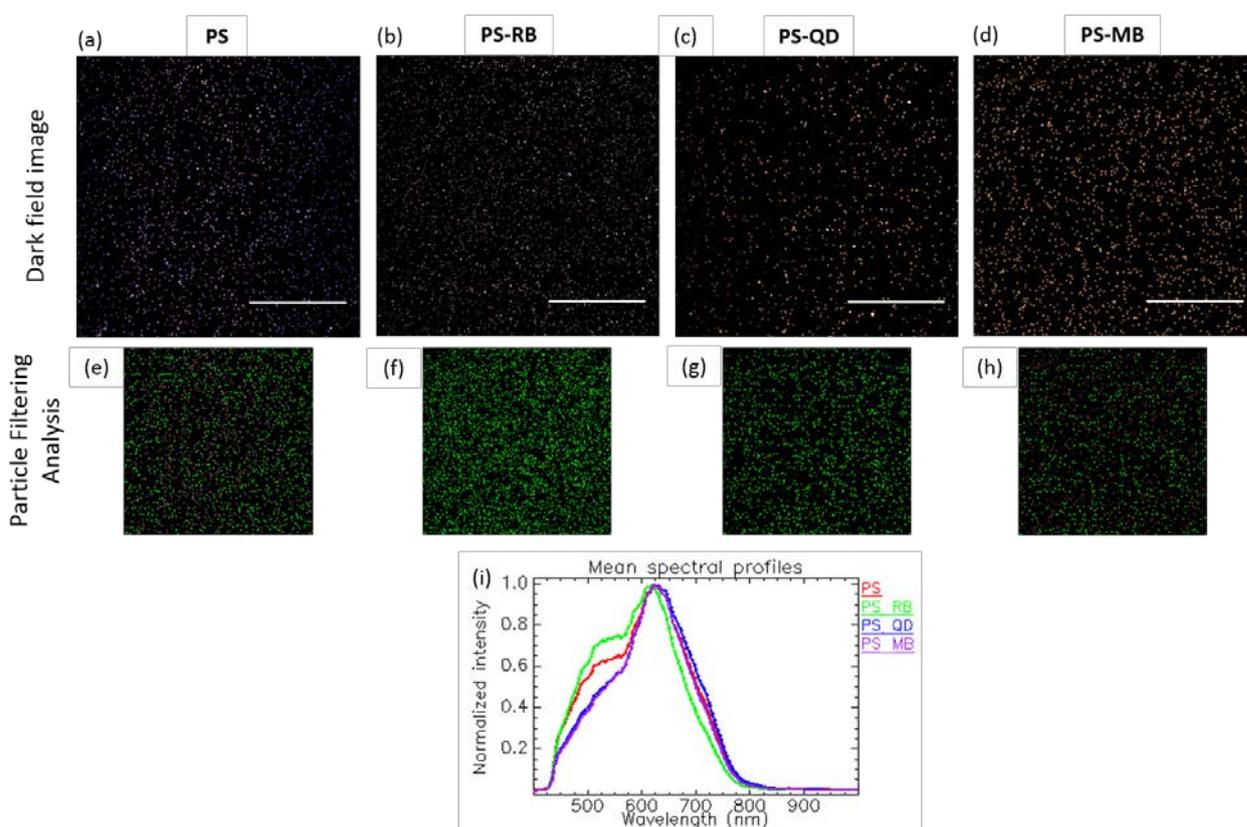
5 Paula Zamora-Perez <sup>1,#</sup>, Dionysia Tsoutsi <sup>1,#,\*</sup>, Ruixue Xu <sup>1</sup> and Pilar Rivera\_Gil <sup>1,\*</sup>

6 <sup>1</sup> Integrative Biomedical Materials and Nanomedicine Lab, Department of Experimental and Health Sciences  
7 (DCEXS), Pompeu Fabra University (UPF), PRBB, Barcelona, Spain;

8 <sup>#</sup> Both authors contributed equally to the manuscript

9 <sup>\*</sup> Correspondence: dionysia.tsoutsi@upf.edu , pilar.rivera@upf.edu ; Tel.: +34 933160918

10  
11 **Figure S1**



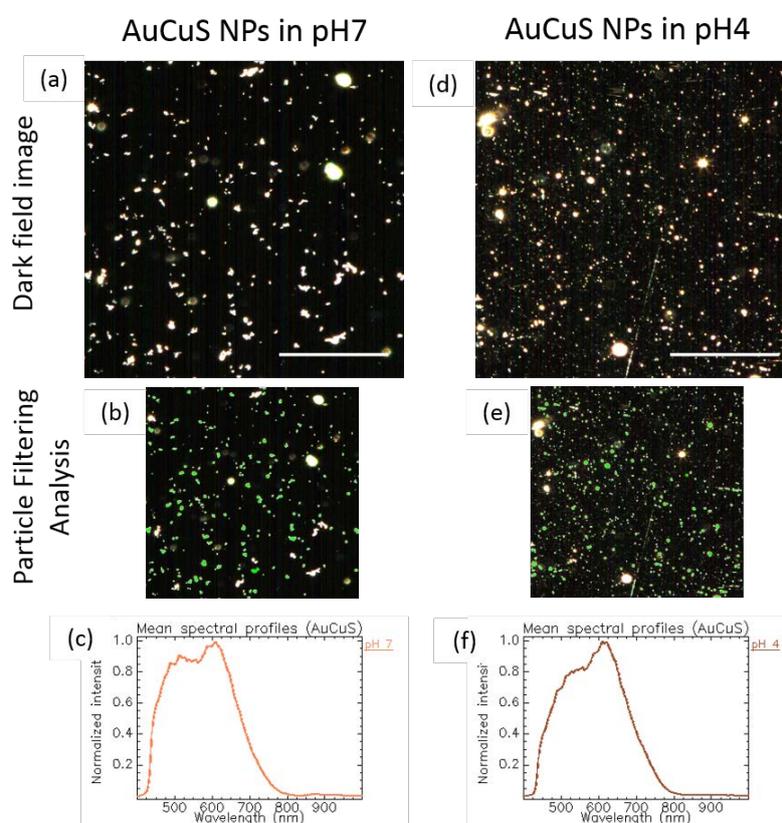
13  
14 **Figure S1:** Mean spectral responses of polystyrene (PS) beads alone and loaded with different cargos  
15 (a-d) dark field images of the bare PS, rhodamine-B-loaded PS (PS-RB), quantum-dot-loaded PS (PS-  
16 QD) and methylene-blue-loaded PS (PS-MB) particles. The particles are well dispersed as can be seen  
17 from the images; (e-h) false-colored dark field images of each sample resulting from the particle  
18 filtering analysis; (i) the mean spectral profiles for each sample calculated from the green false-colored  
19 pixels as shown above (e-h). In specific, we have considered 831 particles (17418 pixels) for PS, 2088  
20 particles (25306 pixels) for PS-RB, 782 particles (12288 pixels) for PS-QD, and finally, 633 particles  
21 (13791 pixels) for PS-MB. The high number of particles considered for the calculation of the  
22 corresponding mean spectra, demonstrates the statistical relevance of the analysis. The scale bar  
23 corresponds to 50  $\mu$ m.

24

25

26

## Figure S2



27

28

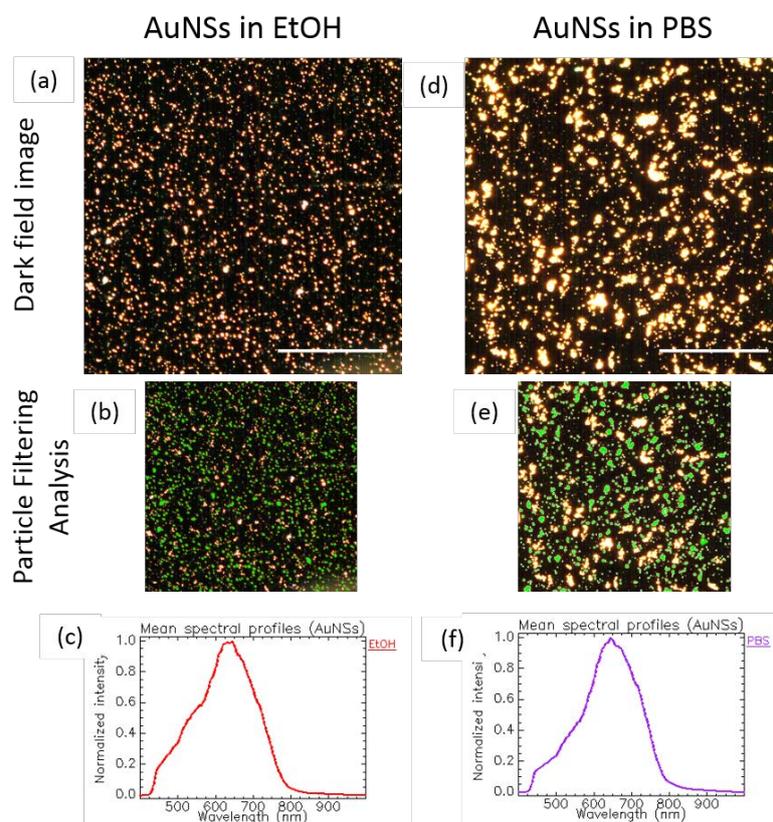
29

30

31

32

**Figure S2:** Hyperspectral analysis of AuCuS NPs at acidic and neutral pH mimicking the intravesicular and cytosolic/extracellular cell areas, respectively. (a-c) False-colored dark field images showing 242 AuCuS NPs in green and the generated mean spectrum at pH 7. (d-f) False-colored dark field images showing 588 particles in green and the generated mean spectrum at pH 4. (scale bar: 50  $\mu\text{m}$ )

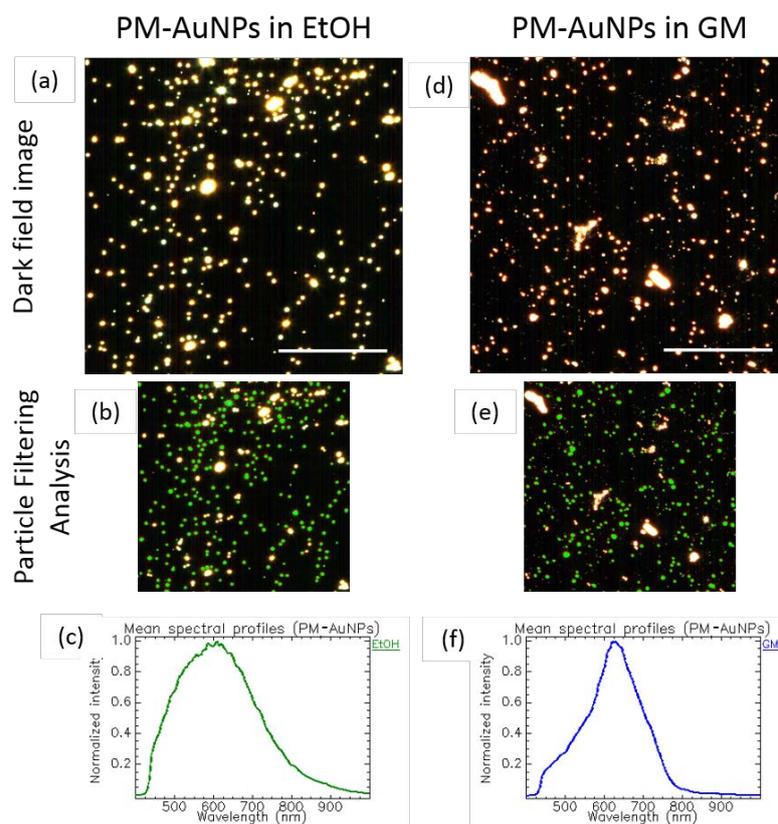
33 **Figure S3**

34

35

36 **Figure S3:** Hyperspectral analysis of AuNSs exposed to different solvent and ionic strength. (a-c)  
37 Green false-colored dark field images containing 987 NPs, and the calculated mean spectrum in  
38 ethanol; (d-f) dark field images containing 882 NPs, and the calculated mean spectrum in PBS. (scale  
bar: 50  $\mu\text{m}$ )

39

40 **Figure S4**

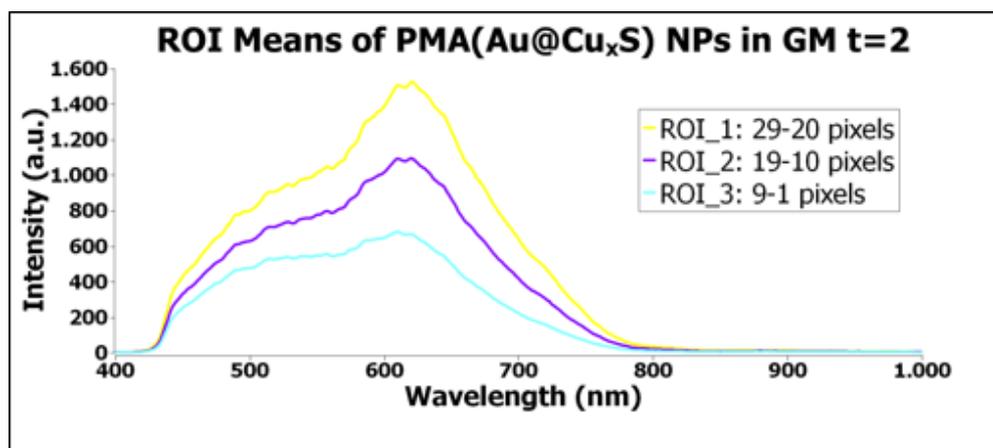
41

42 **Figure S4:** Hyperspectral analysis of the PM-AuNPs varying the solvents' nature and complexity. (a-  
43 c) Green false-colored dark field images containing 253 NPs, and the calculated mean spectrum in  
44 ethanol; (d-f) dark field images containing 254 NPs, and the calculated mean spectrum in GM. (scale  
45 bar: 50  $\mu\text{m}$ )

46

47 **Figure S5**

48



49

50

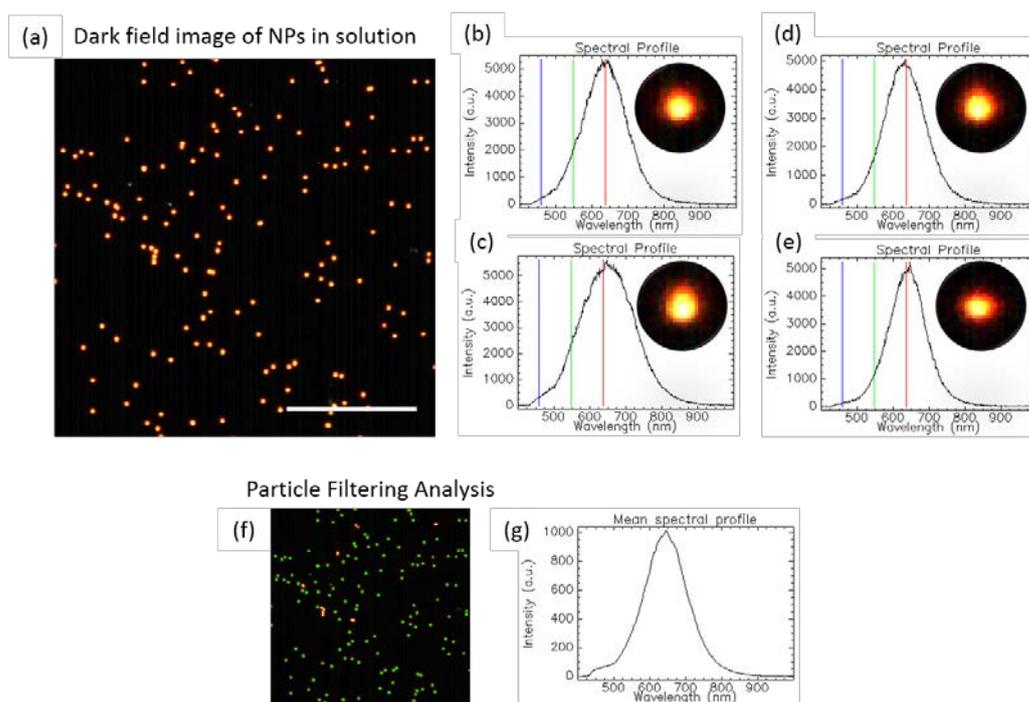
51

52

53

**Figure S5:** Spectral response of gold-copper sulfide NPs (AuCuS NPs) in cell growth media (GM) over time. The mean spectral profiles of 3 different regions of interest (ROI) of AuCuS NPs sorted by the number of pixels represented in the data cube are provided. In specific, the spectral information was obtained after 8 months of exposure of AuCuS NPs to GM.

54

55 **Figure S6**

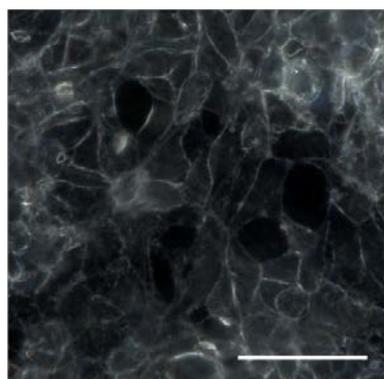
56

57 **Figure S6:** Mean and single particle hyperspectral analysis of 100 nm gold nanospheres in water. (a)  
 58 Dark field image of the NPs in water; (b-e) single spectra at the maximum scattered light intensity  
 59 coming from individual particles randomly selected. The four spectral profiles show similar band  
 60 maximum wavelengths: 645 nm, 645 nm, 630 nm, and 645 nm corresponding to b, c, d, and e plots,  
 61 respectively; (f) false-colored dark field images image showing 136 particles in green; (g) mean  
 62 spectral profile with band maximum at 645 nm. Scale bar: 50  $\mu\text{m}$ .

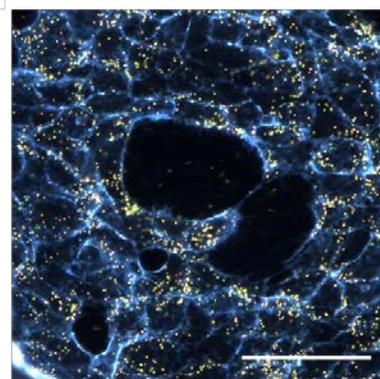
63

64 **Figure S7 - A**

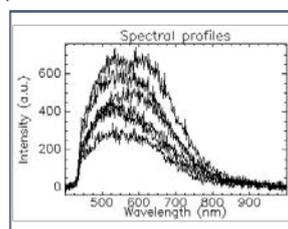
(a) Dark field image of cells without NPs



(c) Dark Field image of NPs inside the cells

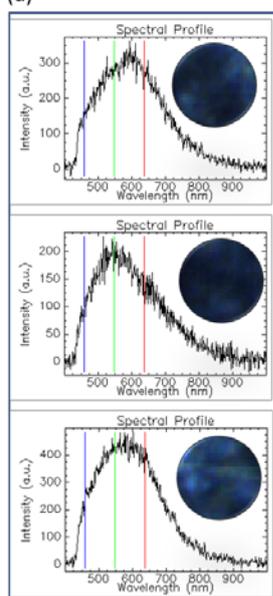


(b) CELL

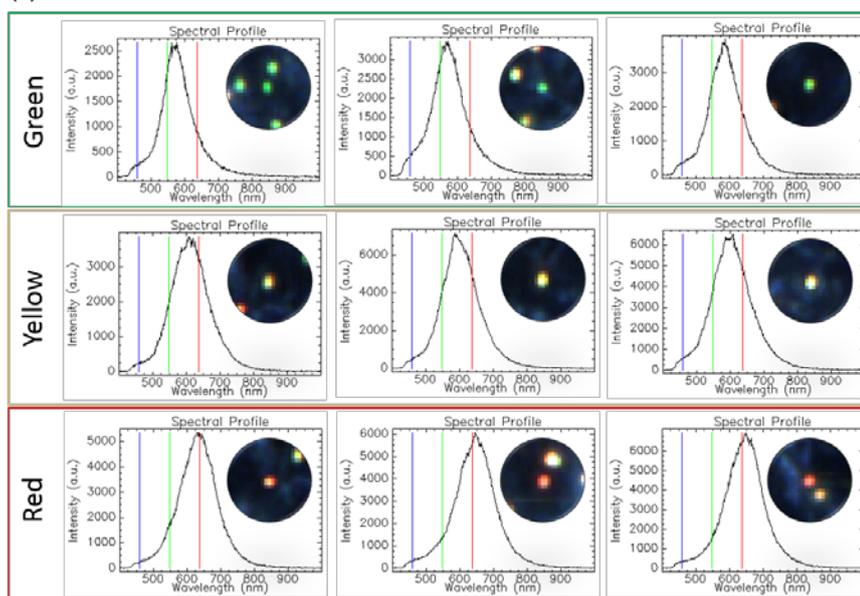


65

(d) CELL



(e) NPs



66

67 **Figure S7:** Spectral behavior analysis of gold nanospheres (100 nm) inside the cell. (a) Dark field image of  
 68 HEK 293T human embryonic kidney cells non-exposed to nanoparticles; (b) Spectral profiles from different  
 69 cell areas randomly selected; (c) Dark field image of HEK 293T cells exposed to AuNPs after 24-hour  
 70 incubation; (d) spectral profiles coming from different cell areas indicating spectral similarity with the  
 71 control cells; (e) spectral profiles of AuNPs categorized upon their different scattering responses in the  
 72 green, yellow and red regions of the visible window. The band maxima in each color category are: 573 nm,  
 73 571 nm, and 582 nm for the green particles, 603 nm, 588 nm, and 588 nm for the yellow, and finally, 633  
 74 nm, 640 nm, and 645 nm for the red. Again, different intracellular conditions provoke changes at the optical  
 75 responses of the nanomaterials. All the plots show the red-green-blue (RGB) bars in which the dark field  
 76 images are based. (Scale bar: 50  $\mu\text{m}$ ).

77 **Figure S7 - B**

78 **Cell maintenance and sample preparation of figure S7:** The HEK293T cell line was provided by the  
 79 laboratory of molecular physiology, department of experimental and health sciences from Pompeu Fabra's  
 80 University. The cells were cultured following ATCC recommendations using: Dulbecco's modified Eagle's  
 81 medium (DMEM) with high glucose content and sodium pyruvate (110 mg/L, n (Gibco, ref. 41966029)  
 82 supplemented with 10% fetal bovine serum (Hyclone, ref. SH30088.02), 200 mM L-glutamine (Hyclone, ref.  
 83 SH30034.01), and 100 U/mL penicillin with 100  $\mu\text{g}/\text{mL}$  streptomycin (Hyclone, ref. SV30010). The passing  
 84 of cells was performed twice per week when reaching 80% confluency, using 0.05% trypsin-EDTA (Gibco,  
 85 ref. 25300062) and Hank's balanced salt solution (HBSS) (Cultek, ref. BE10-547F).

86 HEK293T cells were seeded on coverslips using a 6-well plate and placed overnight in the incubator in an  
87 atmosphere of humidified air with 5% CO<sub>2</sub> at 37°C. 5.6×10<sup>7</sup> citrate-coated gold nanospheres of 100 nm were  
88 added to cells at 75% confluency. After 24 hours of incubation the coverslips were placed into clean  
89 microscope slides and acquisitions were obtained for each sample. Control cells without nanoparticles were  
90 imaged as well.