Electronic Supplementary Material (ESI) for Nanoscale Advances.

Supplementary Information

The impact of macrophage phenotype and heterogeneity on the total internalized gold nanoparticle counts

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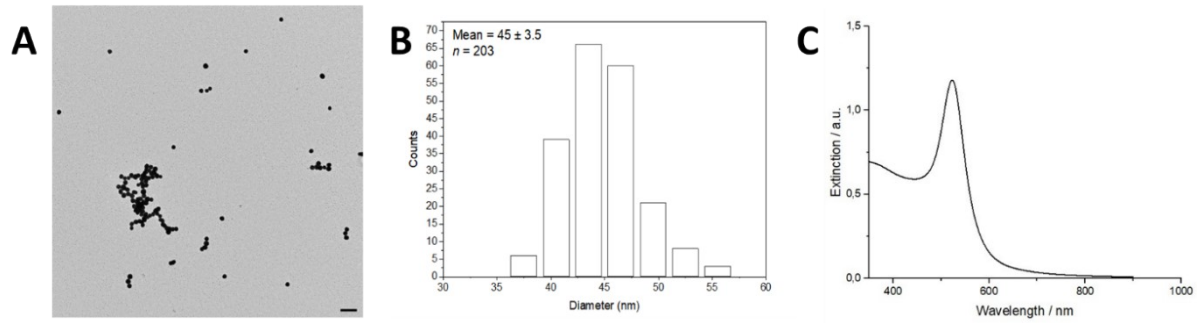

Figure S 1 Representative TEM image (A) of 45 nm AuNPs and the histogram (B) of its sizing data. UV-VIS spectra of AuNPs in H_20. Scale bar: 50 nm

Table S 1 Physicochemical characterization of 50 nm AuNPs.

TEM[1]	**DLS[2]**			
	Milli-Q		**cRPMI[3]**	
d_c [4] (nm)	**d_h [5] (nm)**	**Z [6] (mV)**	**d_h [0] (nm)**	**d_h [48] (nm)**
45 ± 4	57 ± 2	-30 ± 2	50 ± 1	88 ± 3

[1] Transmission electron microscope, **[2]** dynamic light scattering, **[3]** complete Roswell Park Memorial Institute cell culture medium, **[4]** core diameter, **[5]** hydrodynamic diameter, **[6]** zeta potential, hydrodynamic diameter following **[0]** and **[48]** hours of incubation time in cRPMI.

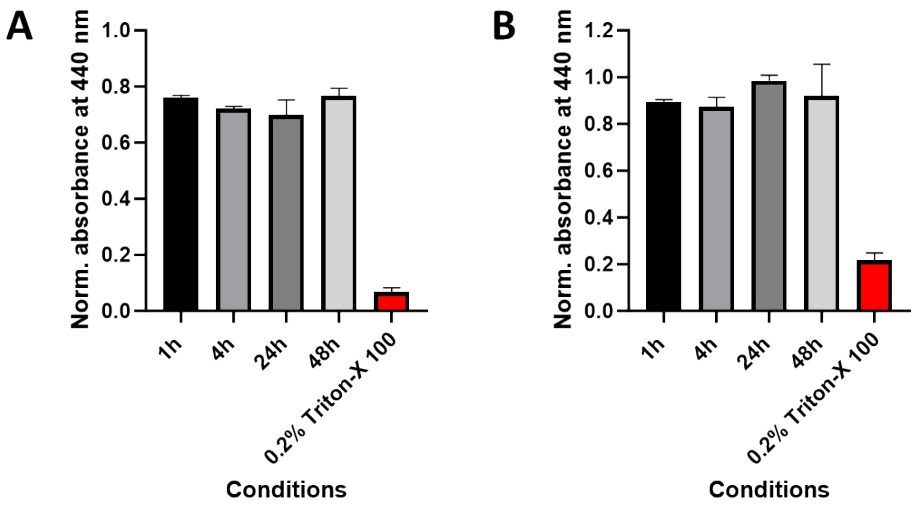

Figure S 2 Cell viability of GM-CSF (A) and M-CSF (B) macrophages was determined by the WST-1 assay following AuNPs exposure. Data was normalized to the negative control (not shown). As a positive control, 0.2 vol.% Triton X-100 was added to the cell culture medium 24 hours before the assay. Data obtained with cells from four independent donors are presented as mean + standard error. Cell culture media, AuNPs, and cells after AuNPs exposure did not interfere with the assay.

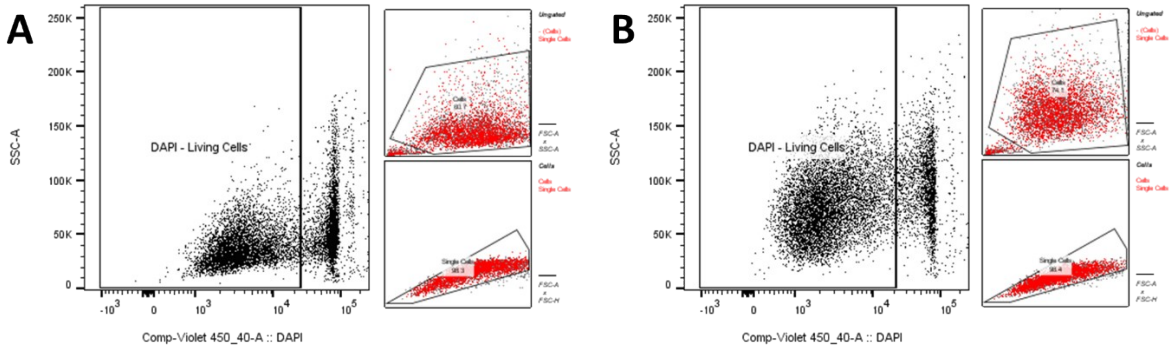

Figure S 3 Representation of the gating strategy employed in cell sorting and analysis. The steps for excluding debris, doublets, and dead cells based on scatter characteristics and fluorescence parameters are illustrated, highlighting the gating regions that isolate single living cells for sorting and cell surface marker analysis. Differences in side scatter intensities in the scatter plot are visible between 1h (A) and 48h (B) of AuNPs exposure.

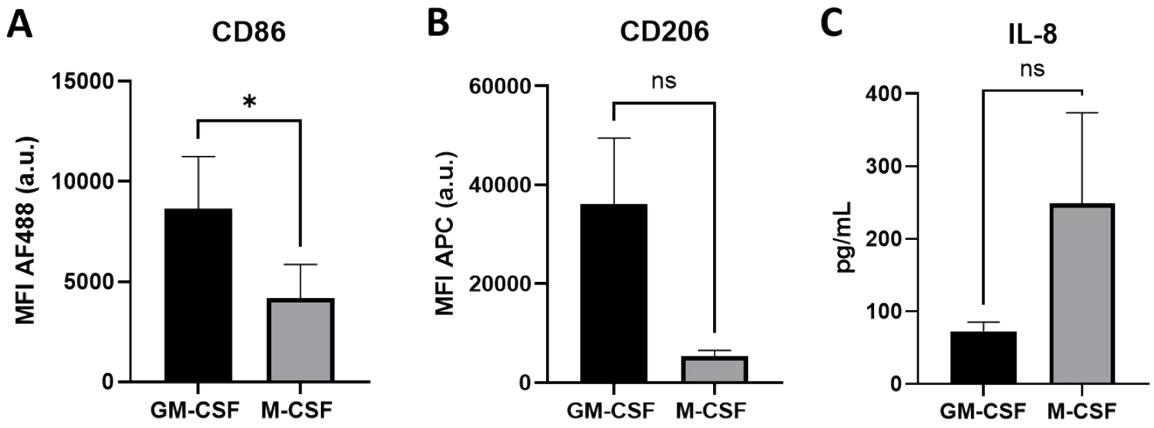

Figure S 4 Expression levels of CD86 and CD206 were assessed using flow cytometry, while the secretion levels of IL-8 were measured by ELISA in both GM-CSF and M-CSF macrophage control cells. The flow cytometry analysis revealed that GM-CSF macrophages exhibited higher expression levels of CD86 (A) and CD206 (B) compared to M-CSF macrophages, with the difference in CD86 expression being statistically significant. Additionally, M-CSF macrophages showed a tendency to express higher levels of IL-8 (C) under basal conditions compared to GM-CSF macrophages. Statistical significance between conditions was determined using the paired student t-test with a significance level of *p < 0.05. Analyses were performed using Prism 8.

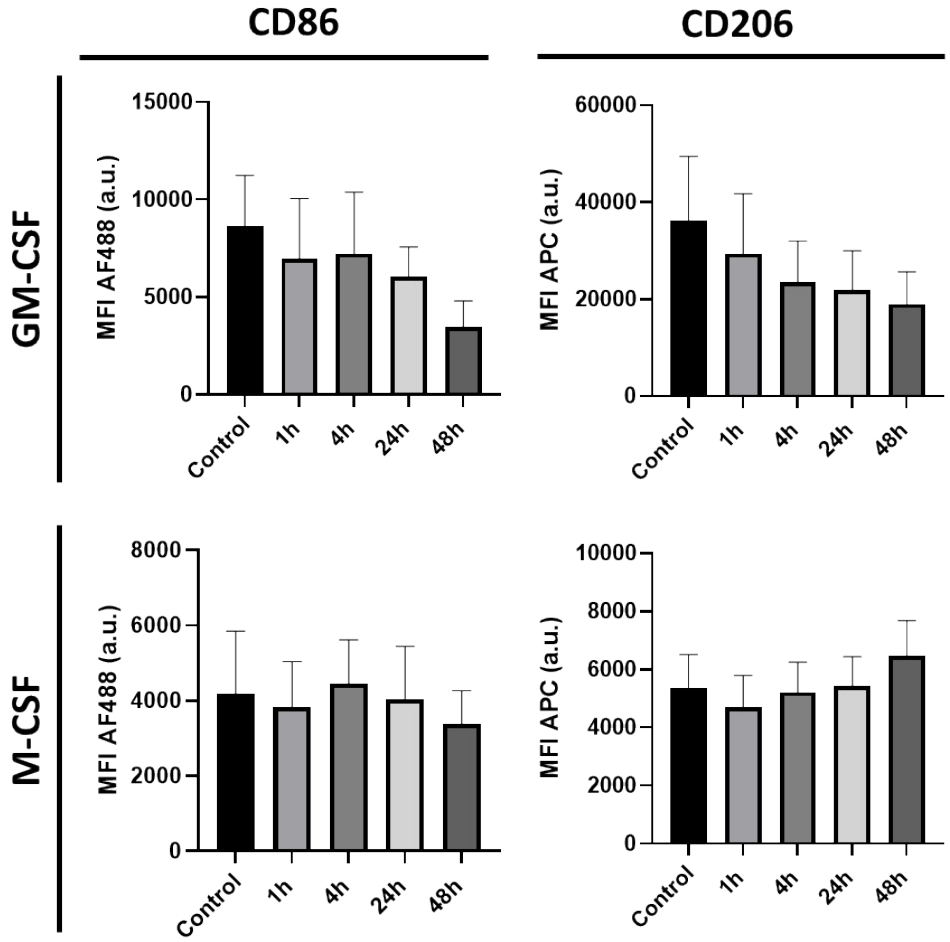

Figure S 5 Cell surface marker expression levels of CD86 and CD206 in GM-CSF and M-CSF macrophages exposed to AuNPs. Statistical significance between conditions was determined using a one-way ANOVA followed by Dunnett's multiple comparison test with a significance level of *p < 0.05. Analyses were performed using Prism 8.

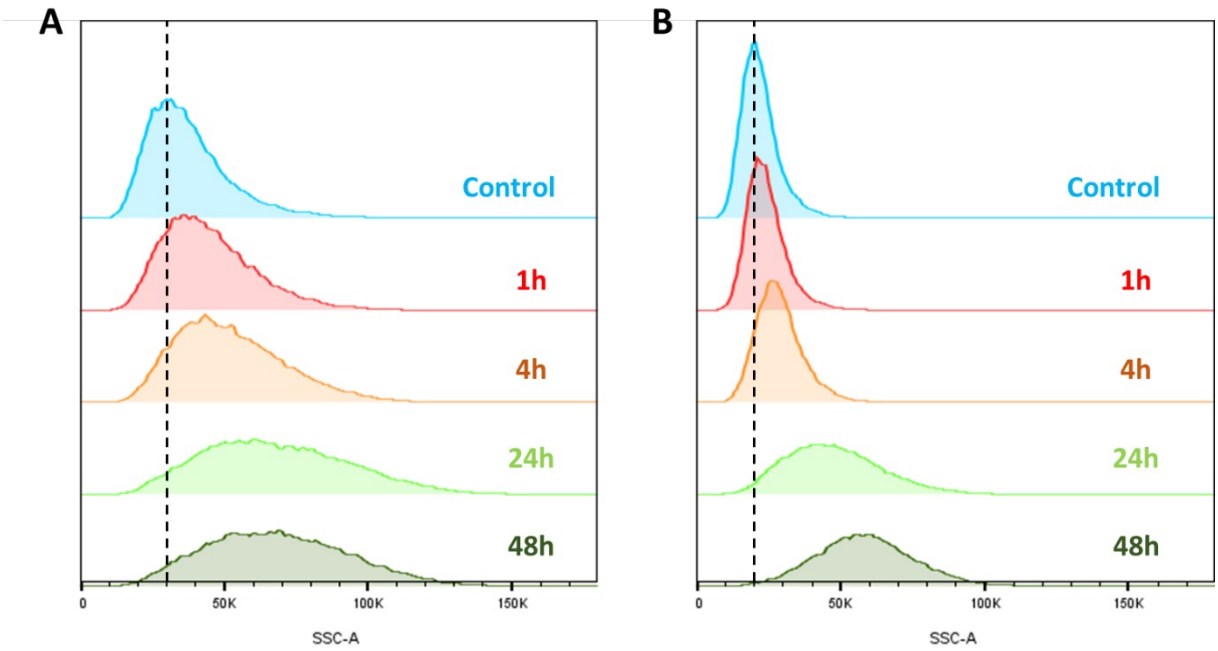

Figure S 6 Histograms generated from flow cytometry analysis visually represent data distribution. The half-offset display showcases the complete distributions of GM-CSF (A) and M-CSF (B) macrophages exposed to AuNPs for varying durations of 1, 4, 24, and 48 h, including the control group.

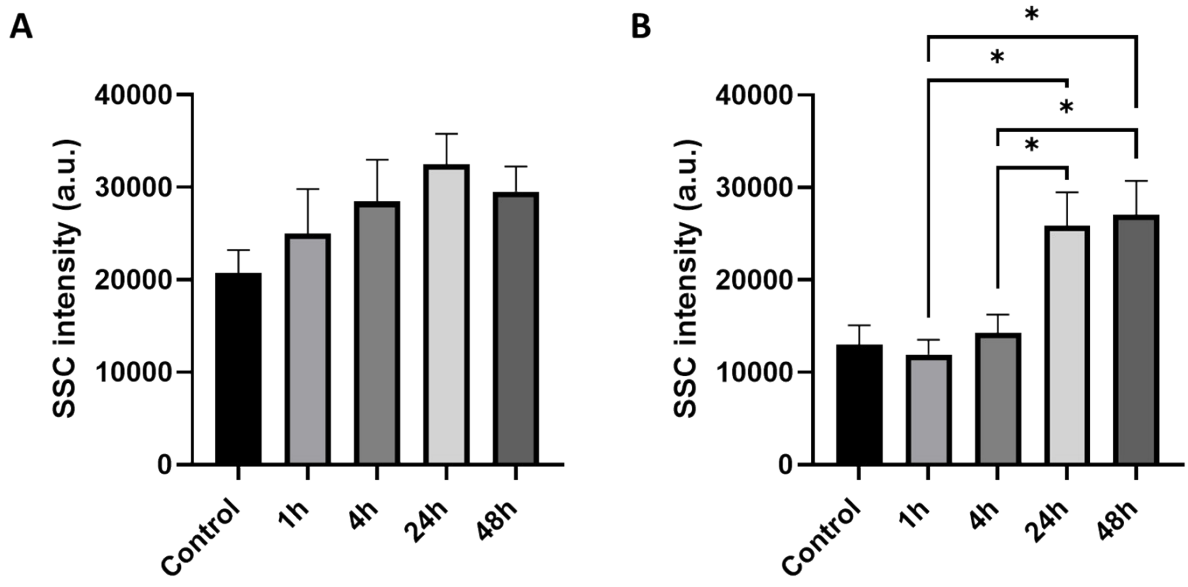

Figure S 7 Standard deviations (SD) of the SSC intensity distributions obtained by flow cytometry of GM-CSF (A) and M-CSF (B) macrophages presented as a column bar graph (*n*=4). SD values of the SSC intensities were obtained with the statistical tool in the software Flowjo. Statistical significance between conditions was determined using a one-way ANOVA followed by Holm-Šídák's multiple comparisons test with a significance level of *p < 0.05. Analyses were performed using Prism 8.

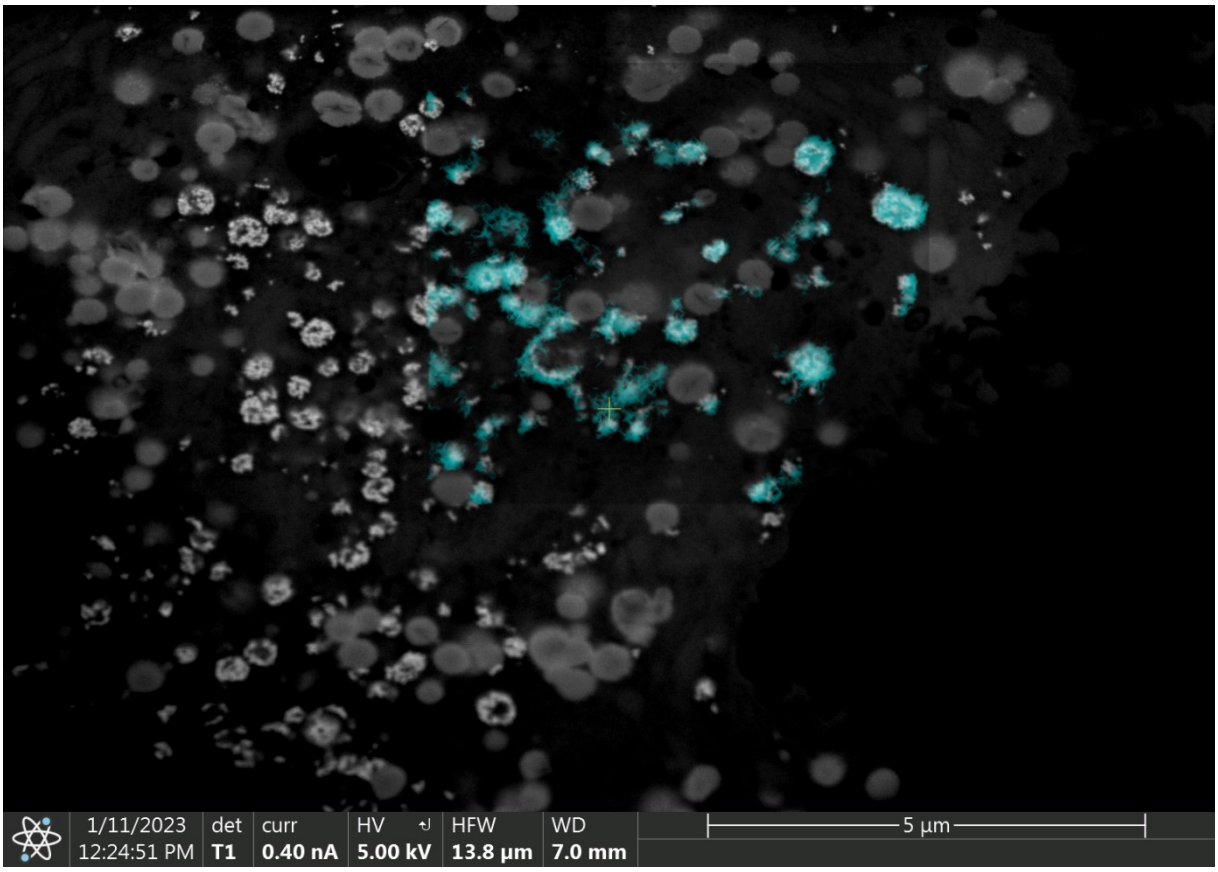

Figure S 8 Overlay of a back-scattered electron (BSE) image displaying a cross-sectional view of a macrophage post-exposure to AuNPs and energy-dispersive spectroscopy (BSE) map highlighting the presence of the element gold (Au).

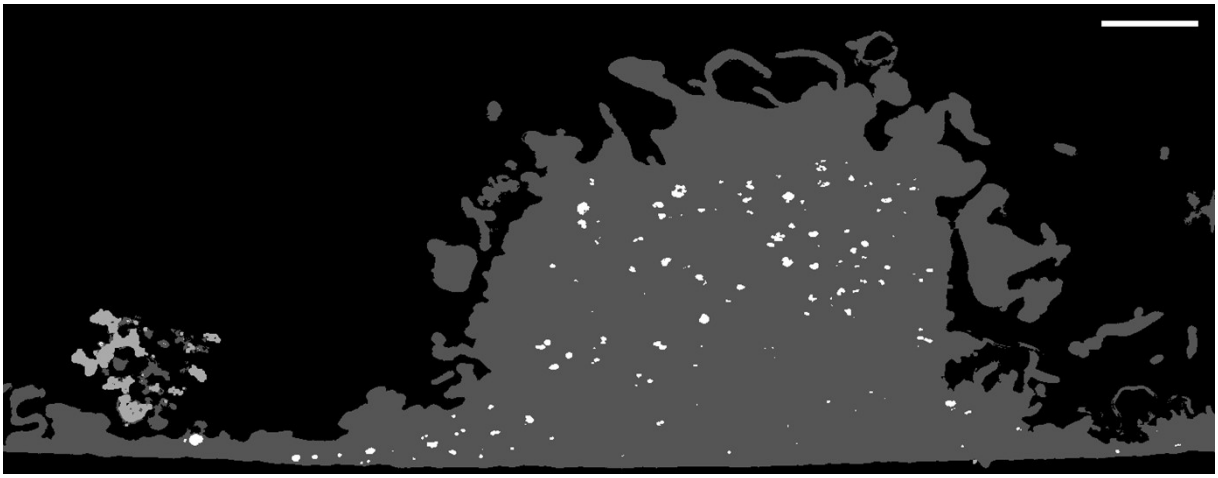

Figure S 9 A representative segmented image to determine the cardinal count of internalized AuNPs. The image is divided into distinct pixel values representing the background (black), cell (gray), internalized (white), and extracellular (light gray) AuNPs. This segmentation technique allowed the visualization and quantification of AuNPs within the cellular context and provided insights into their distribution. Scale bar: 20 µm

```
Datafolder = 'F:/Henry/Tomographies to analyze/MCSF/analysis 48h/t1 segmented/';
Safefolder = 'NPs split/';
first= '0';
last = '1069';
runOpen = 1; //Runs an Open filter on the image
// Program
print("\\Clear");
setBatchMode(true);
for (slice = first; slice <=last; slice++){
if(slice < 1000){
if(slice < 1000){
count_ = '0' + d2s(slice,0);
}
if(slice < 100){
count_ = '00' + d2s(slice,0);
}
if(slice < 10){
count_ = '000' + d2s(slice,0);
}
} else {
count = d2s(slice,0);
}
print("Processing File number " + slice);
//SEM-Multi-Detector-Image_T1_3---SliceImage---001.view1024_Simple Segmentation
open(Datafolder + "SEM-Multi-Detector-Image_T1_3---SliceImage---001.view"+count+"_Simple Segmentation.tif");
//open(Datafolder + "SEM-Multi-Detector-Image_T1_3---SliceImage---003.view"+count+"_Simple Segmentation.tif");
rename("origin");
perform_split(count, runOpen);
}

function perform_split (count, runOpen){
selectWindow("origin");
selectWindow("Particles outside");
run("Divide...", "value=255");
run("Multiply...", "value=2");
selectWindow("Particle inside");
run("Divide...", "value=255");
run("Multiply...", "value=3");

run("Merge Channels...", "c1=[cell mask] c2=[Particle inside] c3=[Particles outside] create");
Stack.setDisplayMode("color");
Stack.setChannel(1);
run("Grays");
setMinAndMax(0, 3);
Stack.setChannel(2);
run("Grays");
setMinAndMax(0, 3);
Stack.setChannel(3);
run("Grays");
setMinAndMax(0, 3);
Stack.setDisplayMode("composite");
run("Stack to RGB"); //this will stretch the values from 0-3 to 0-255
run("8-bit");
// rest the pixel values to 0, 1, 2 and 3
run("Divide...", "value=85");
rename("final");
selectWindow("particle mask");
close();
selectWindow("origin");
close();
selectWindow("Composite");
close();
saveAs("Tiff", Datafolder+Safefolder+"NPsSegmented"+count+".tif");
close();
}
run("Duplicate...", "title=[cell mask]");
run("Multiply...", "value=20");

//set threshold 60
setThreshold(55, 64);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Fill Holes"); //check this

selectWindow("origin");
run("Duplicate...", "title=[particle mask]");
run("Multiply...", "value=20");

setThreshold(75, 65535);
run("Convert to Mask");

imageCalculator("Multiply create", "cell mask","particle mask");
selectWindow("Result of cell mask");
rename("Particle inside");
//run("Open");
selectWindow("cell mask");
run("Invert");
imageCalculator("Multiply create", "cell mask","particle mask");
selectWindow("Result of cell mask");
rename("Particles outside");

if (runOpen == 1){
run("Open");
}
// SLIT IN 3 GROUPS: cell, inside NP and outside NP
selectWindow("cell mask");
run("Invert");
run("Divide...", "value=255");
```

Script S 1 ImageJ/FIJI script written to classify AuNPs in segmented SEM images into extra- and intracellular localization.