**Electronic Supplementary Information**

**3D multiplexed immunoplasmonics microscopy**

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# FIGURES

## Optimization of HS-PEG concentration

HS-PEG was diluted from 10 mg mL$^{-1}$ to 2.5 µg mL$^{-1}$ in water. Suspensions of citrate-capped NPs (320 µL) in DI water were treated with an aqueous solution of HS-PEG (35.5 µL) or water (35.5 µL, control reaction). The mixtures were vortexed and kept for 1 h at 4 °C. Subsequently, they were treated with either water (39.5 µL) or a 10% (w/v) aqueous NaCl solution (39.5 µL). The mixtures were kept at 4 °C for 30 min. An aliquot of each sample (320 µL) was placed in a 96-well plate for analysis by UV-visible-NIR spectroscopy.

Stable PEGylated NPs in 1% NaCl were obtained with at least 5 µM HS-PEG for AuNSs or 40 nm x 92 nm AuNRs, and at least 40 µM HS-PEG for AgNSs or 53 nm x 107 nm AuNRs (Fig. S1). In the experiments described in this paper, HS-PEG was added at a concentration of 40 µM for AgNSs, AuNSs and 40 nm x 92 nm AuNRs, and 200 µM for 53 nm x 107 nm AuNRs. PEGylation of NPs triggered a plasmon band's red-shift of 6 nm for AgNSs, 3 nm for AuNSs, 5 nm for 40 nm x 92 nm AuNRs and 4 nm for 53 nm x 107 nm AuNRs.
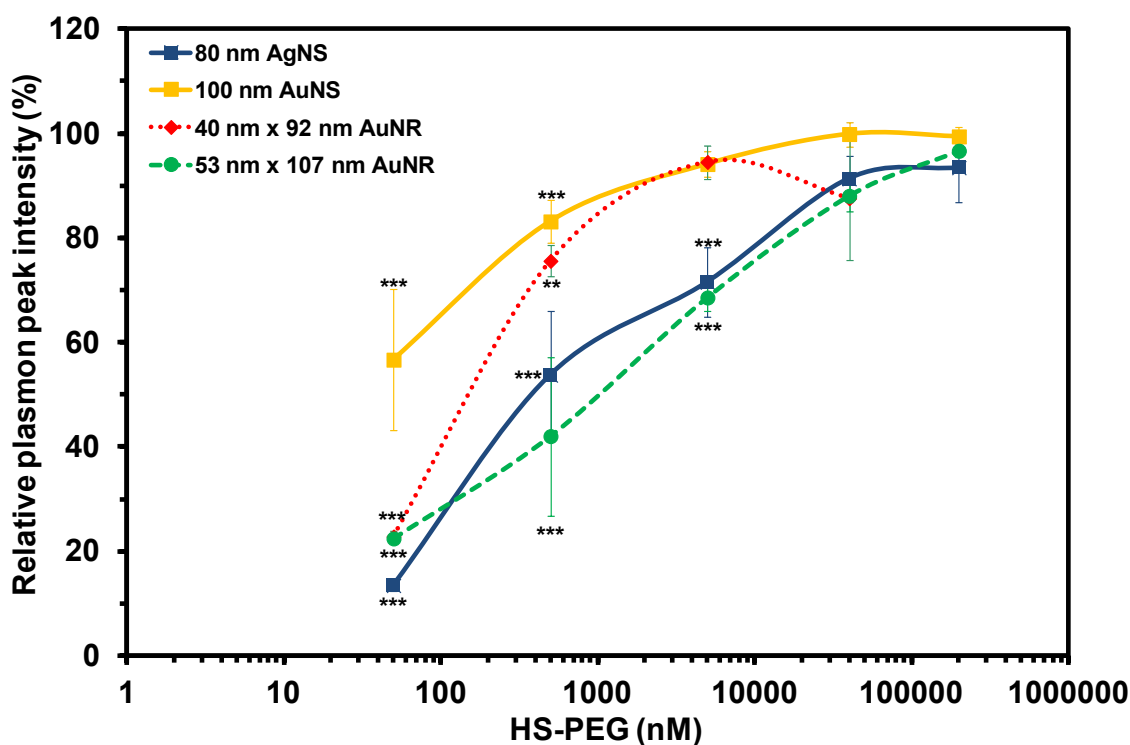


**Fig. S1** Normalized plasmon peak absorbance measured by UV-visible-NIR spectroscopy of NPs functionalized with increasing amounts of HS-PEG (5 kDa, 50 nM to 200 µM) incubated in 1% NaCl for 30 min. Other independent experiments gave similar results: AgNSs (n = 7), AuNSs (n = 11) and AuNRs (40 nm x 92 nm, n = 3; 53 nm x 107 nm, n = 4). Results are expressed as means ± standard deviation (SD). Statistically significant differences are indicated for each treatment by **p < 0.01 and ***p < 0.001 in comparison to citrate-capped NPs incubated in water.
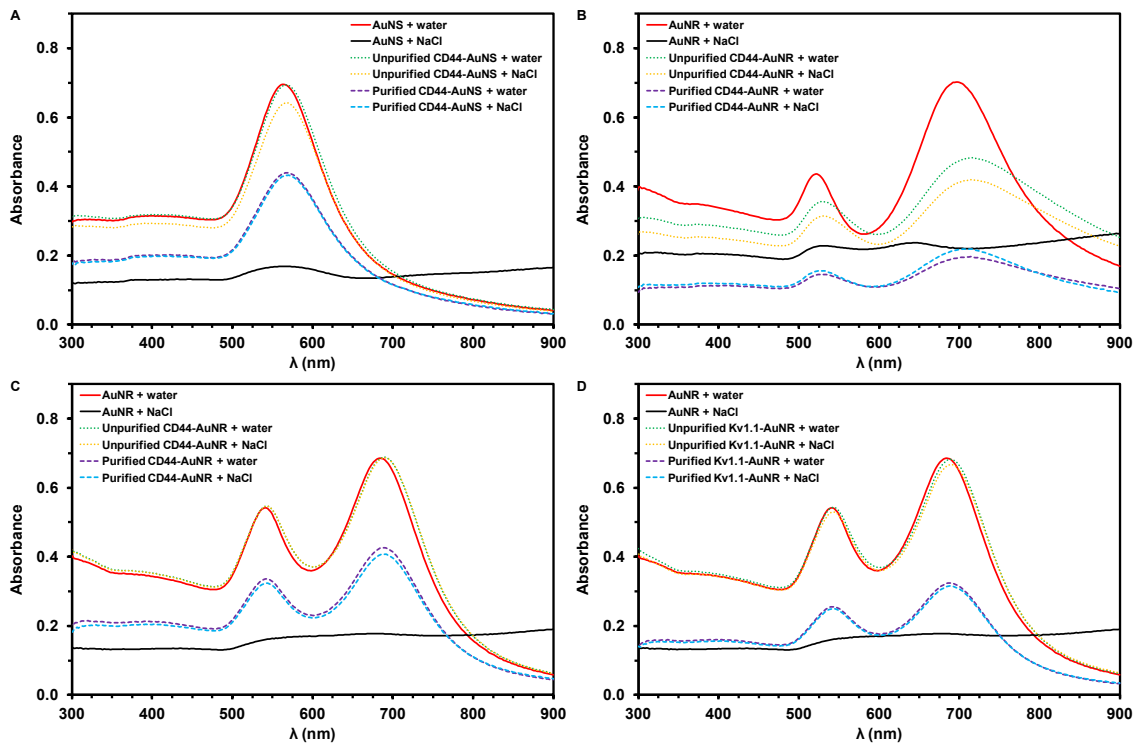
**Fig. S2** Representative experimental UV-visible-NIR spectra of bare NPs and fNPs with OPSS-PEG-Abs and HS-PEG: CD44-AuNSs (A, n = 8), CD44-AuNRs (40 nm x 92 nm; B, n = 2), CD44-AuNRs (53 nm x 107 nm; C, n = 2), and $K_V1.1$-AuNRs (53 nm x 107 nm; D, n = 4). Samples before purification or after centrifugation and resuspension in phenol red-free DMEM were incubated for 30 min in water or 1% NaCl.

**Darkfield microscopy of NPs in cellular environment**

Light elastically scattered by the plasmonic NPs and the cells was detected by standard darkfield microscopy with filters commonly used in fluorescence microscopy (ET-DAPI, ET-GFP and ET-DsRED).[20]

Citrate-capped AgNSs, AuNSs and AuNRs attached non-specifically to the light-scattering cellular environment and the substratum (Fig. S3A–H). With the combination of source, fluorescence filters, NPs and camera, 100 nm AuNSs were more easily observed than 80 nm AgNSs or 40 nm x 92 nm AuNRs. These latter were more difficult to visualize with the darkfield microscopy set-up since only the light at wavelengths under 650 nm was collected while the longitudinal AuNRs' scattering peak was located around 700 nm. PEGylated AgNSs, AuNSs and AuNRs attached weakly to the cells, thus indicating efficient prevention of their non-specific attachment to the cells (Fig. S3I–N).

Since CD44 is strongly expressed by MDA-MB-231 cells while 661W cells do not express this biomarker (Fig. 6A, D), AgNSs and AuNRs were functionalized with Abs anti-CD44 to verify that the protocol[20] developed for AuNSs would allow efficient functionalization of AgNSs and AuNRs, resistance to aggregation in saline environment and selective labelling of targeted cells. Selective cell targeting was observed with the three types of CD44-NPs and the best contrast was observed with AuNSs with a plasmon peak around 570 nm (Fig. S3O–T). Scanning electron microscopy previously revealed that CD44-AuNSs were mostly single and attached 115 times more to targeted MDA-MB-231 cells than to non-targeted 661W cells.[20] EGFR-AuNSs and $K_V1.1$-AuNRs were then prepared with the same functionalization protocol and incubated with the cells (Fig. S3U–X). The detection of fNPs targeting low-expressing EGFR and $K_V1.1$ biomarkers (Fig. 6B,C,E,F) was difficult with standard darkfield microscopy in the light-scattering cellular environment. Thus, RLM was used in the following experiments to improve the contrast of fNPs over the cells.
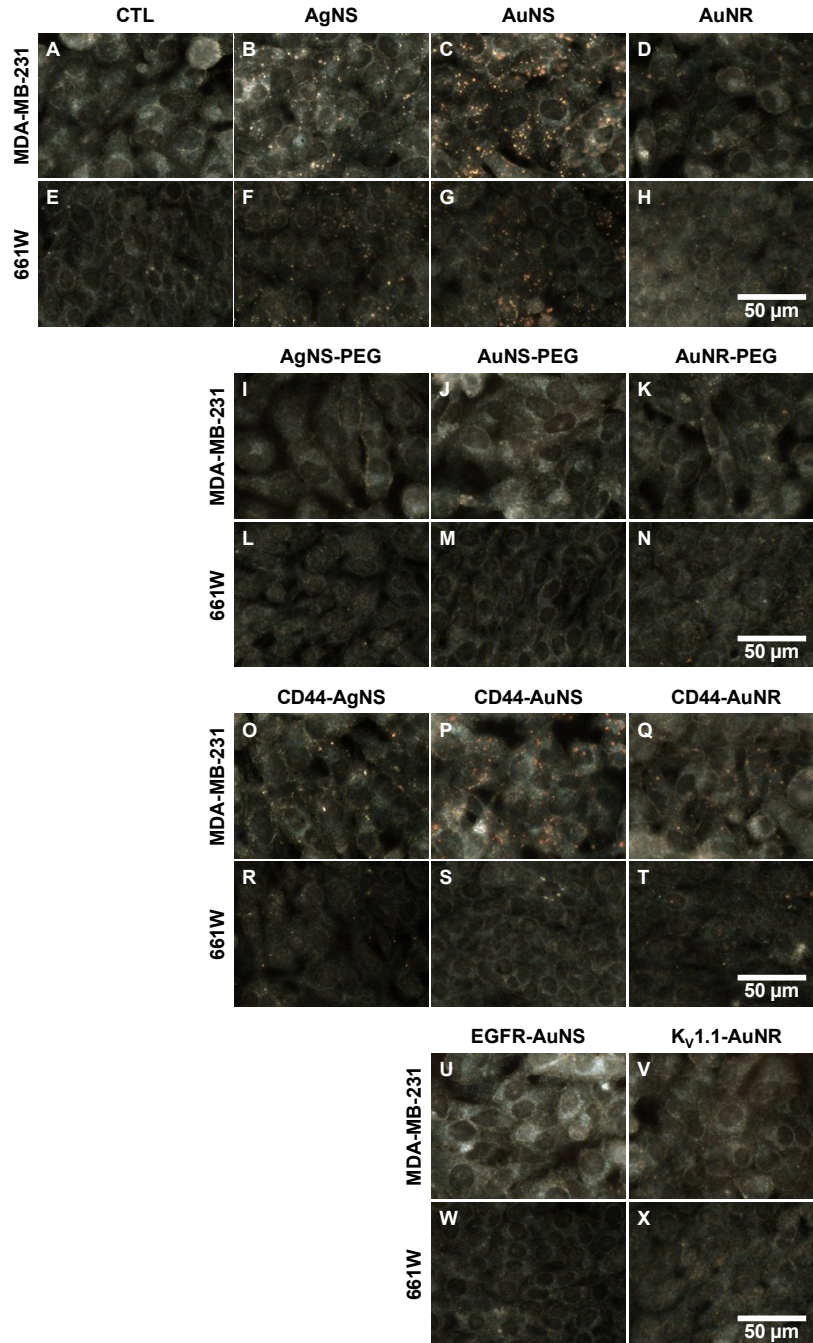
**Fig. S3** Darkfield microscopy of CD44$^+$ EGFR$^+$ K$_V$1.1$^+$ MDA-MB-231 and CD44$^-$ EGFR$^-$ K$_V$1.1$^+$ 661W cells incubated for 3 h in DMEM/10% FBS/1% PS without (A,E) or with 8 µg mL$^{-1}$ citrate-capped NPs [AgNSs (B,F), AuNSs (C,G), AuNRs (40 nm x 92 nm; D,H)], PEGylated NPs [AgNSs (I,L), AuNSs (J,M), AuNRs (K,N)], or fNPs [CD44-AgNSs (O,R), CD44-AuNSs (P,S), CD44-AuNRs (Q,T), EGFR-AuNSs (U,W) and K$_V$1.1-AuNRs (V,X)]. Exposure time of 30 ms for each fluorescence filter. Objective 60× with additional 1.5× magnification. Representative images were obtained from three independent experiments.
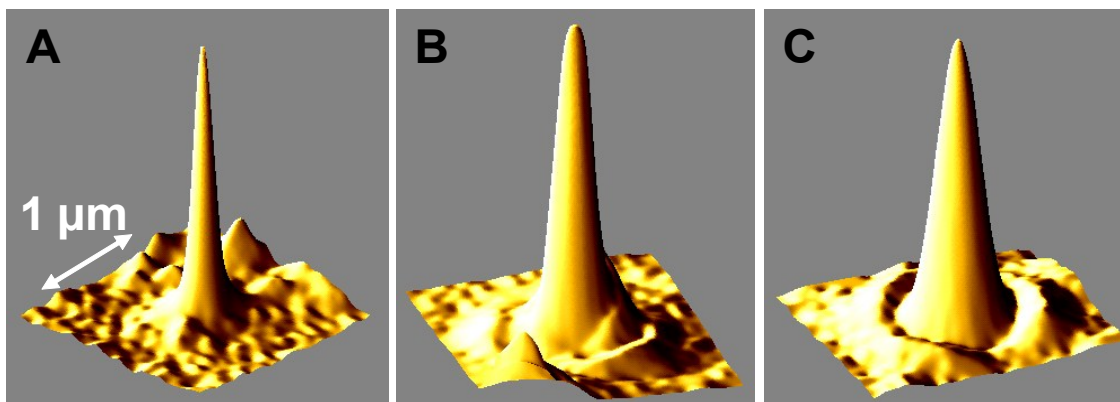
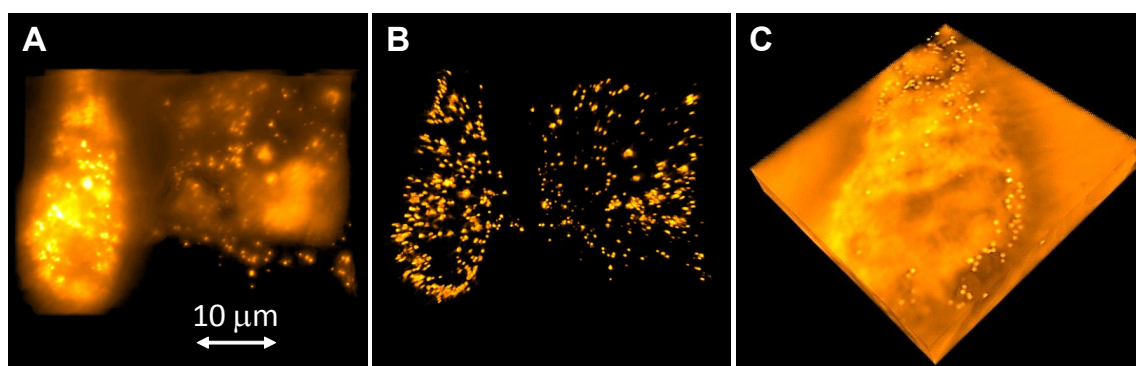**Fig. S4** Experimental PSFs for AgNS at 500 nm, AuNS at 580 nm and AuNR (40 nm x 92 nm) at 700 nm.



**Fig. S5** Example of image treatment for immunoplasmonics by using the proposed RLM set-up. (A) 3D image of EGFR-AuNSs on CD44$^+$ EGFR$^+$ K$_V$1.1$^+$ MDA-MB-231 cells obtained with 100 nm step z-scans and objective 100×. (B) Deconvolution of the image using experimental PSF. (C) 3D position of EGFR-AuNSs obtained by local maximum filtering and 3D Object Counter (ImageJ) combined with cell image.
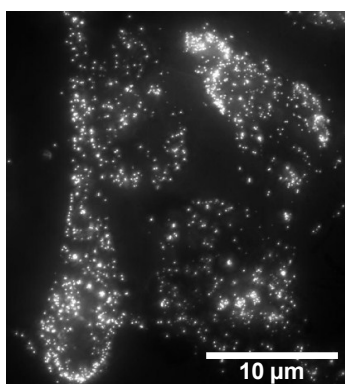


**Fig. S6** 2D image of EGFR-AuNSs on CD44$^+$ EGFR$^+$ K$_V$1.1$^+$ MDA-MB-231 cells obtained by z-stacks' integration from Video S1.
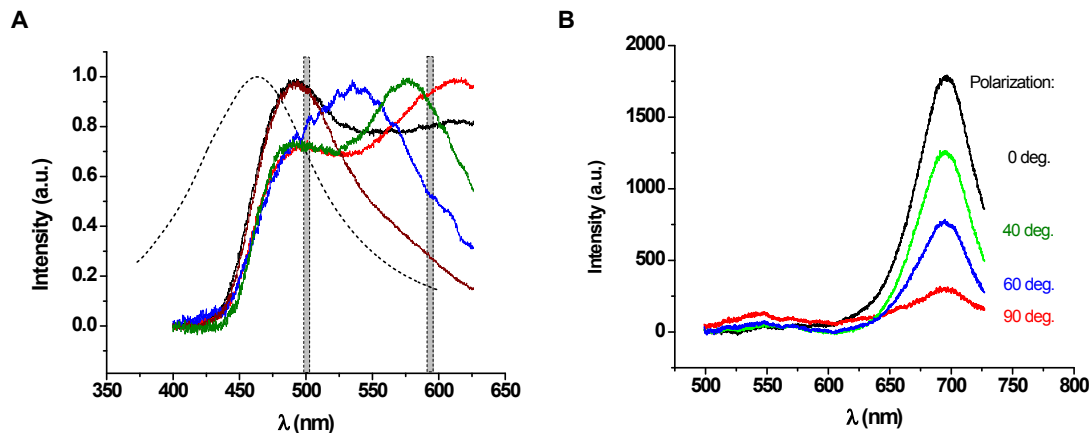
**Fig. S7** (A) Normalized experimental scattering spectra of AgNSs aggregates. (B) Experimental backscattering intensity dependence of AuNRs (40 nm x 92 nm) for different polarized illumination angles.
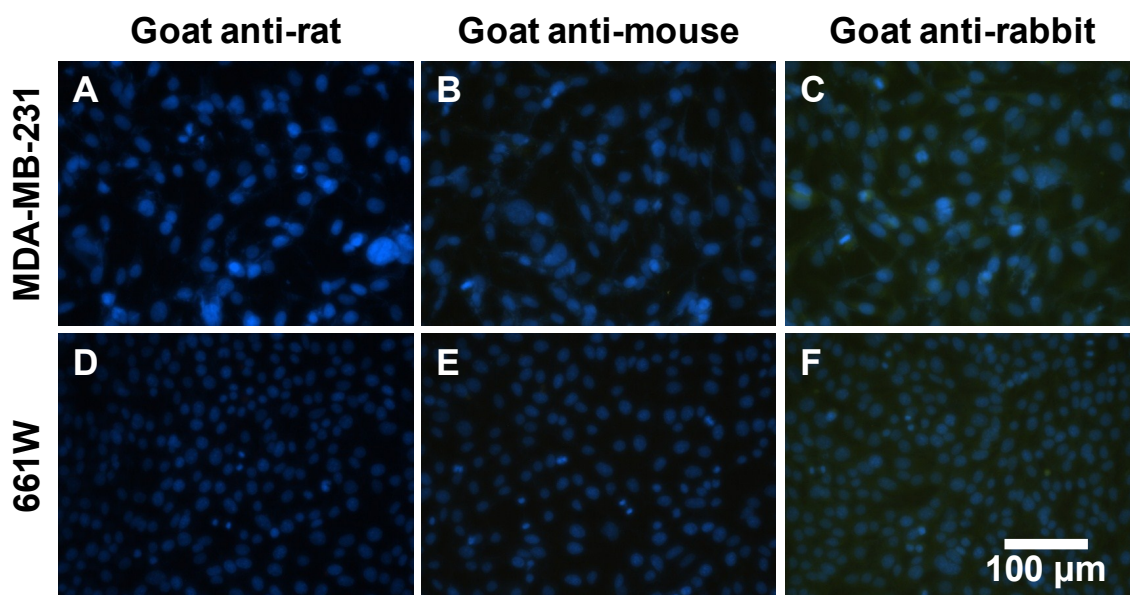


**Fig. S8** Immunofluorescence of CD44$^+$ EGFR$^+$ K$_V$1.1$^+$ MDA-MB-231 and CD44$^-$ EGFR$^-$ K$_V$1.1$^+$ 661W cells without primary Abs was detected with fluorescently-labelled secondary Abs: red-emitting Cy3 conjugated to goat anti-rat IgG Abs (A and D, exposure time of 80 ms) or green-emitting Alexa Fluor 488 dye conjugated to goat anti-mouse (B and E, exposure time of 500 ms) or goat anti-rabbit (C and F, exposure time of 500 ms) IgG Abs (Objective 20× with additional 1.5× magnification). Cell nuclei were stained with DAPI (blue). Representative images were obtained from four independent experiments.

**VIDEO**
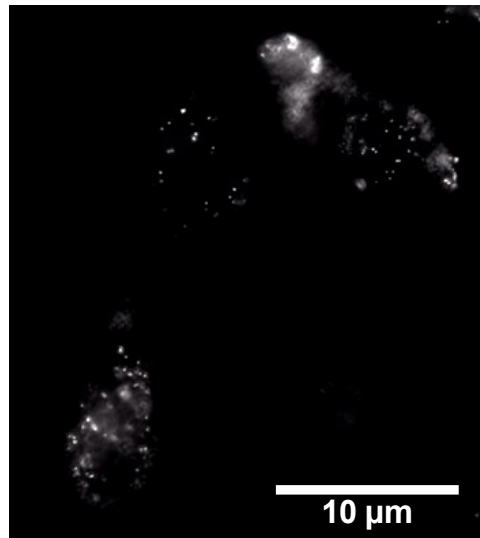


**Video S1** Experimental z-scan 2D image of EGFR-AuNSs on CD44$^+$ EGFR$^+$ K$_V$1.1$^+$ MDA-MB-231 cells by using RLM.

**TABLE**

**Table S1** Physical characteristics of citrate-capped NPs.

| NP | Supplier | Catalog number | Lot | Size | Aspect ratio | Concentration | | | pH | Zeta potential | Plasmon peak(s) | Experimental plasmon peak(s) | Diameter |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **Information provided by the supplier** | | | | | | **UV-visible-NIR spectroscopy** | **Mie theory** |
| | | | | (nm) | | ($10^9$ NPs/mL) | (µg/mL) | (µM) | | (mV) | (nm) | (nm) | (nm) |
| AgNS | TED Pella | 84060-80 | DMW0155 | 79.3 ± 9.5 | 1.0 | 8.0 | 22 | 204 | 8 | -45 | 455 | 458 | 78 |
| AuNS | | A11-100-CIT | SPC359C | 100 | 1.0 | 5.7 | 50 | 254 | 7 | -46 | 566 | 564 | 95 |
| AuNR | Nanopartz | A12-40-700-CIT | F3542 | 40 x 92 | 2.3 | 31.0 | 60 | 305 | 7 | -35 | 520 (transverse) and 700 (longitudinal) | 521 (transverse) and 697 (longitudinal) | |
| AuNR | | A12-50-700-CIT | F3614 | 53 x 107 | 2.0 | 16.0 | 60 | 305 | 7 | -35 | 541 (transverse) and 700 (longitudinal) | 541 (transverse) and 684 (longitudinal) | |