Plasmonic Labeling of Subcellular Compartments in Cancer Cells: Multiplexing with Fine-tuned Gold and Silver Nanoshells

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Supporting Information

MATERIALS

Reagents and consumables. Tetrachloroaurate ($HAuCl_4$), silver nitrate ($AgNO_3$), L-ascorbic acid ($C_6H_8O_6$), Tetraethyl orthosilicate 98%, (3-aminopropyl)trimethoxysilane 97%, polyvinylpyrrolidone (MW-55000), Ammonium hydroxide (NH_4OH) 28%, sodium borohydride ($NaBH_4$), formaldehyde 37% (CH_2O), Triton X-100, n-hexanol and propidium iodide were purchased from Sigma-Aldrich. Opti-MEM® reduced serum media, Dulbecco's modified Eagles medium, Fetal Bovine serum and Trypan Blue 0.4% solution were purchased from Thermo Fisher Scientific; custom-made NLS peptide (PKKKRKV) was purchased from Pharmaster Laboratories, Anti-IGFR antibody (sc-713) and Palloidin CruzFluor® 488 were obtained from Santa Cruz Biotechnology; 0.22 μm cellulose acetate syringe filters were obtained from Sterlitech. Notice that filters from different brands can lead to small variations in the synthesis because the quality of the cellulose used in the filters vary according to different standards in different Countries. High quality filters are recommended.

Instruments. Barnstead Nanopure Diamond Ultra-Pure Water system, FisherBrand FB11201 Ultrasonic bath, Fisher Scientific Accuspin® 17 microcentrifuge, Fisher Scientific Isotemp® magnetic stirring plate, Brookhaven ZetaPals® Particle Analyzer, Varian Cary® 50 UV-Vis-NIR spectrophotometer, JEOL JEM-1400 Transmission Electron Microscope (All size determinations from TEM had 200 particles counted), Cytoviva Dual Mode Fluorescence/Hyperspectral Dark Field Microscope.

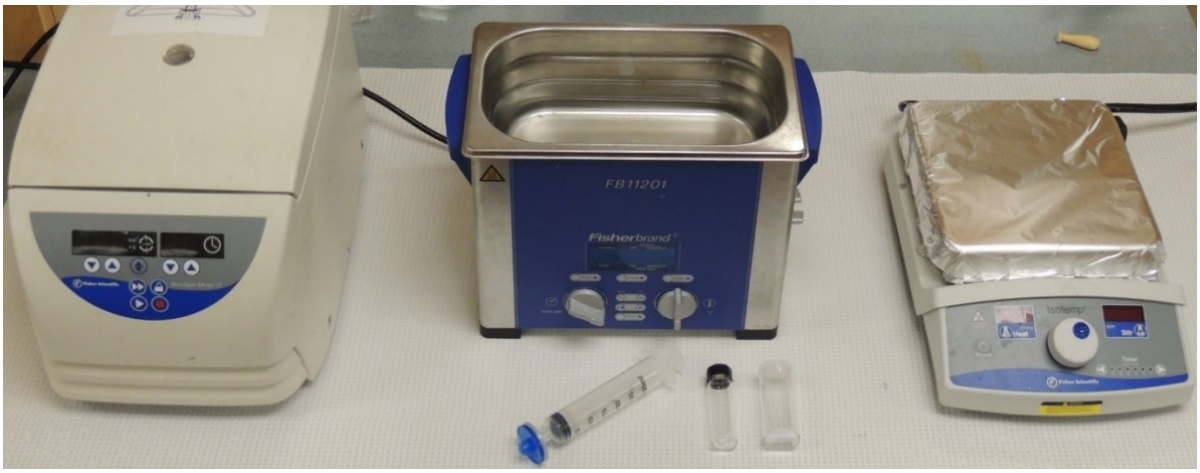

Figure SI-1. Only simple labware is required for the synthesis of the nanoshells. Left to right: Microcentrifuge, ultrasonic bath, 0.22 µm syringe filter, vials, stir bars and stirring plate.

METHODS

All the synthetic methods described in this work take place at room temperature. Cell incubations happen at 37^{0}C in an incubator at 5% CO_2.

One-batch synthesis and functionalization of aminated silica nanoparticles. In a clean glass vial, 7.5 mL of cyclohexane, 1.7 grams of Triton X-100 and 1.8 mL of 1-hexanol are placed under magnetic stirring at 800 rpm. In a separate vial, 25 µL of NH_4OH (28%) and 500 µL of ultrapure water are mixed to constitute the aqueous phase of the reverse microemulsion system. This aqueous solution is then added to the organic phase under stirring and the vial is capped. Micelles are allowed to form and stabilize for one hour. 100 µL of TEOS is then added to the vial. Sequential additions of 100 µL of TEOS are made every six hours for bigger SiO_2 sizes (See Table SI-1). After the reaction time has elapsed, the vial is opened and NH_3 is allowed to escape for two hours before functionalization. The pH should then reach a range of 8-9 before silanization is performed. pH paper or pH strips can be used in this step. Performing the silanization without lowering the pH may result in aggregated samples. In this case 600 µL of a 24 nM APTMS solution in cyclohexane is added to the reaction vial and kept under stirring for 5 minutes. 15 mL of anhydrous ethanol are then added to break the micelles. The sample is centrifuged at 5000g/5min. Three to five cleaning cycles are repeated to remove excess amounts of reagents and the sample is finally resuspended in a total 30 mL of ethanol before being placed under sonication for 15 minutes for a more vigorous cleaning that will remove

adsorbed molecules from the surface of the silica. Centrifugations happen at 10000g/10 now and five vigorous cleaning cycles take place before the sample can be resuspended in ethanol and stored. During the cleaning process the colloid will change from a cloudy and flocculated aspect into a stable and semitransparent suspension. The aminated silica colloids used here were kept on a benchtop at room temperature for up to 4 months. The number of silica nanoparticles per sample can be determined through a simple process as described in Table SI-1.

Table SI-1. Parameters for the synthesis of SiO_2 nanoparticles of different diameters.

100 μL-TEOS additions*	Reaction time after last TEOS addition (hours)	Resulting diameter and standard deviation (nm)
1	24	50 ± 2.5
3	12	63 ± 2.8
3	36	72 ± 2.5
4	24	80 ± 2.3

* After the first TEOS, subsequent additions happen every 6 hours. For instance, 3 means the first TEOS addition, following 6 hours waiting, then another TEOS addition, another 6 hours waiting and a final 100 μL addition.

Fabrication of small (2.1 ± 0.3 nm) gold nanoparticles. In a 600 mL beaker, 3.425g of PVP (MW-55000) are slowly dissolved in 190 mL of ultrapure water. 4.075 mL of a 20 mM $HAuCl_4$ solution (aq.) is then added to the beaker. Formation of the small gold nanoparticles is achieved by turning the stirring speed up to 1500 rpm and quickly adding 57 mL of a freshly prepared 5.24 mM $NaBH_4$ solution to the vortexing mixture. A quick change in color from light yellow to dark brown is verified. The resulting colloid is kept under stirring at 1500 rpm for 15 minutes before being ready for use. We recommend that this colloid be used fresh as some growth can be seen in the particles after a period of 24-48 hours.

Nanoislands formation. Typically, 500 μL of aminated silica colloid (containing approximately $2x10^{12}$ particles) is sonicated and 24.5 mL of freshly prepared small-gold colloid is placed under gentle stirring at 300 rpm. The

aminated silica (in ethanol – there is no need to transfer the silica into water for this step) is then added to the vial containing the gold nanoparticles and the reaction mixture is kept under stirring for 3 hours. Saturation of the silica surface can be verified by UV-Vis spectroscopy as demonstrated in our previous work.[25] The formed nanoislands colloid can then be cleaned by centrifugation in water 5 times at 10000g/10min. The final product can be resuspended in 500 μL of ultrapure water and kept in the fridge for up to one month. With longer storage periods the gold nanoparticles on the surface of the silica tend to aggregate. This will negatively affect the shell growth as the coating process will become inhomogeneous.

Shell growth. It is important to notice that the appropriate amount of nanoislands to be used in this step can change according to the desired shell thickness. Less nanoislands will produce thicker shells and vice-versa. In order to provide readers with a reference, considering the biggest SiO_2 core (80nm), 1 μL of nanoislands colloid resulted in a 13 nm gold shell; and 5 μL of nanoislands colloid resulted in a 15 nm silver shell. **Plating solution.** Typically, 13 mL of a 0.15 mM of $HAuCl_4$ or $AgNO_3$ is prepared in a glass vial and placed under stirring at 200 rpm. 25 μL of NH_4OH (aq., 28%) are added and after 5 minutes the resulting solution is filtered through a 0.22 μm cellulose acetate filter into a clean vial. For the growth of **gold nanoshells**, the appropriate amount of nanoislands is added to the gold plating solution under stirring at 200 rpm, the stirring speed is increased to 800 rpm and 90 μL of a 10mM ascorbic acid solution is added to the mixture. Stirring is kept for 5 minutes and after that the sample is left undisturbed for 30-45 minutes before being cleaned by centrifugation-resuspension in ultrapure water at 5000g/5min. For the growth of **silver nanoshells**, 25 μL of formaldehyde (aq., 37%) is added to the silver plating solution at 200 rpm. The stirring speed is then increased to 800 rpm and the appropriate amount of nanoislands is rapidly added to the mixture. Stirring is kept for 10 seconds and after that the sample is left undisturbed for 30-45 minutes before being cleaned by centrifugation in ultrapure water at 5000g/5min. While the aminated silica core holds strong colloidal and chemical stability over long periods of time (Figure SI-3), we recommend that the bioconjugation step be performed with freshly prepared nanoshells, due to the lower chemical stability of the silver nanoshells.

Sample-to-sample variations regarding reaction time for the shell growth may happen in this step of the process, depending on factors such as shell thickness, number of particles per sample and environmental conditions such as

temperature variations. In order to monitor the progress of the growth, UV-Vis-NIR spectroscopy measurements can be taken over time as to establish optimal reaction times.[25]

Bioconjugation to NLS peptide. 1 mL of a 40 mM solution of the peptide was placed under vigorous stirring at 600 rpm. 1 mL of a silver nanoshells suspension – Ag(18nm)@SiO_2(50nm), containing approximately $5x10^9$ particles was added dropwise. The mixture was kept under stirring for 12 hours before being cleaned by centrifugation-resuspension in ultrapure water at 5000g/10min. Prior to incubation, the NLS-coated silver nanoshells are resuspended in 1 mL of Opti-MEM® reduced serum media.

Bioconjugation to anti-IGFR antibody. Gold nanoshells – Au(18nm)@SiO_2(72nm), were prepared by resuspending approximately $5x10^9$ particles in 500 μL of PBS. 500 μL of antibody solution (60 μg/mL) in PBS is placed under vigorous stirring and the nanoshells suspension is added dropwise. The mixture is kept under stirring for 40 minutes and cleaned by centrifugation-resuspension in PBS at 10000g/10min. The sample is finally resuspended in 1 mL of PBS prior to incubation.

Bioconjugated samples can be kept in the fridge at 10^0C if necessary before being used for cell-labeling experiments. We point to the fact though that not all biomolecules will remain optimally active at this temperature. In such case, bioconjugated samples should be prepared immediately before use. In our experiments all bioconjugated samples were prepared one day before use.

Cell culture and plasmonic labeling. MCF-7 breast human cancer cells were plated onto sterile coverslips in six-well plates, and maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum to a confluency of approximately 30%. Cell growth and labeling with nanoshells took place in an incubator at 37°C and 5% CO_2. Cell cultures were washed three times with PBS before incubations. Single incubation time for the Ag-NLS sample (Fig. 6a) was 3 hours. Single incubation time for Au-IGFR nanoshells (Fig. 6b) was 30 minutes. Sequential incubation for the multiple-target experiment was done first with the Au-IGFR sample, and after 30 minutes the cells were washed three times with PBS and the Ag-NLS sample was added for 3 hours. After three PBS washings, the cells were then fixed with 4% paraformaldehyde and mounted on microscope slides for analysis.

IMAGES

Ultramonodisperse non-aggregated aminated silica

Figure SI-2 depicts the silica samples used in this work as well as dynamic light scattering measurements of the bare and aminated colloids (functionalized with increasing amounts of APTMS).

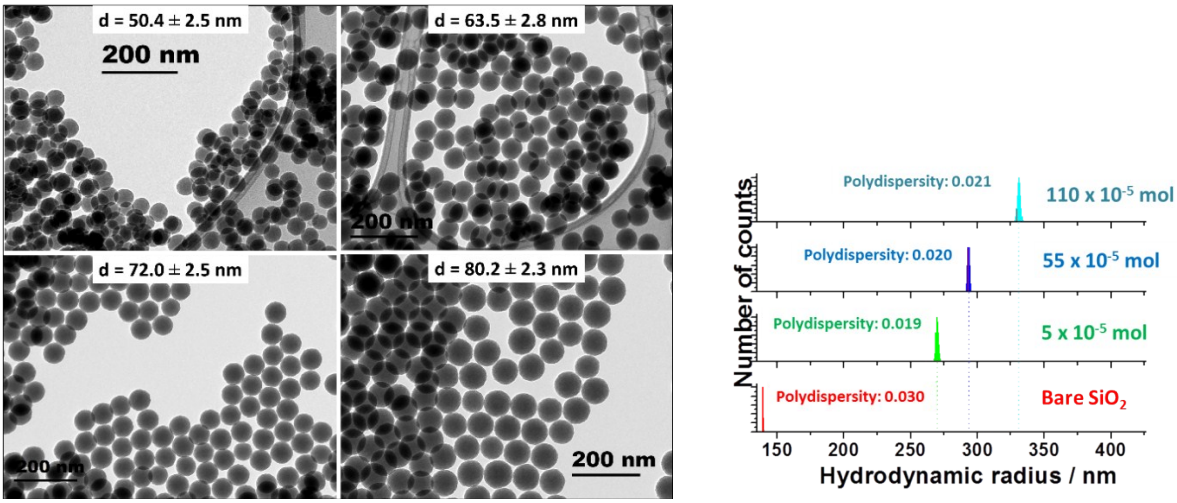

Figure SI-2. Ultramonodisperse SiO_2 samples as produced by the reverse microemulsion approach. Low polydispersity indexes are achieved through this method, indicating the successful fabrication of ultramonodisperse samples, and the absence of aggregation.

Colloidal and chemical stability

Samples can be kept on the benchtop for several months. A stability study was performed with a 10-month old aminated silica sample. The old sample was imaged by TEM before (Figure SI-3a, b) and after being used for the synthesis of nanoislands (Figure SI-3c).

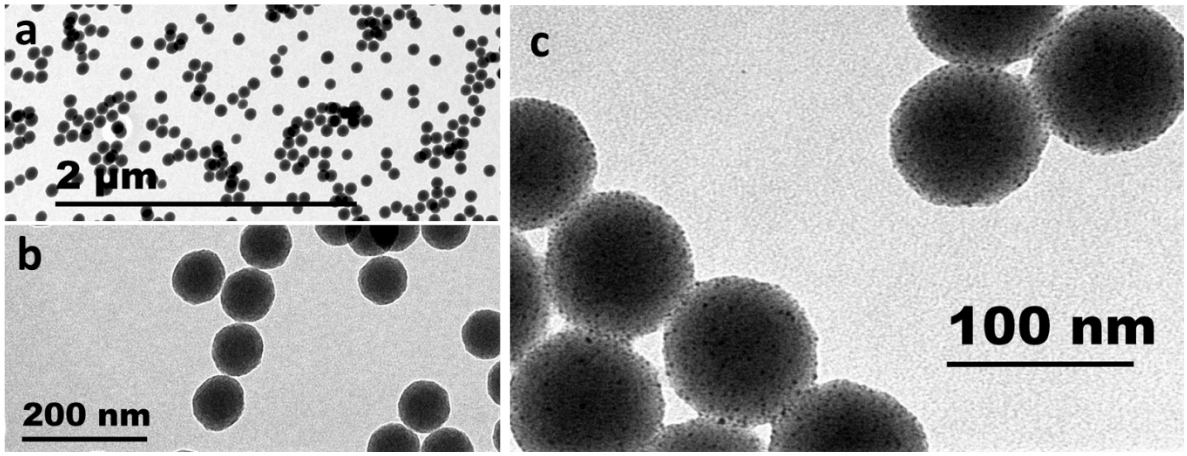

Figure SI-3. Long-term stability of aminated silica samples. TEM images of a 10-month old silica colloid before (a, b) and after nanoislands synthesis (c).

These images attest the great long-term stability of these colloids. SI-3a, b show the colloidal stability in the preserved shape and uniformity of the particles. SI-3c shows the chemical stability of the surface functionalization with APTMS.

Amount of added nanoislands and shell thickness.

The appropriate amount of nanoislands to be added to the plating solution can change according to the desired shell thickness. In order to provide readers with a reference, considering the largest SiO_2 core (80nm), 1 µL of nanoislands colloid resulted in a 13 nm gold shell; and 5 µL of nanoislands colloid resulted in a 15 nm silver shell. The amount of particles per milliliter in the silica colloids can also be estimated by a simple method. 1 mL of a silica colloid whose diameter has been determined by TEM, can be dried and have its mass measured. This mass can be converted into a volume through a simple cross-multiplication method using the density of SiO_2. By using the mean diameter – as determined by TEM, it is possible to calculate the volume of a single silica particle. After that, dividing the total volume (as found by cross-multiplication) by the volume of a single silica particle will give the estimated number of SiO_2 particles per milliliter. The silica samples produced in this work have particles ranging from 2.43×10^{12} to 4.05×10^{12} particles per milliliter.

Figure SI-4 displays examples of the gold or silver nanoshells fabricated through this method.

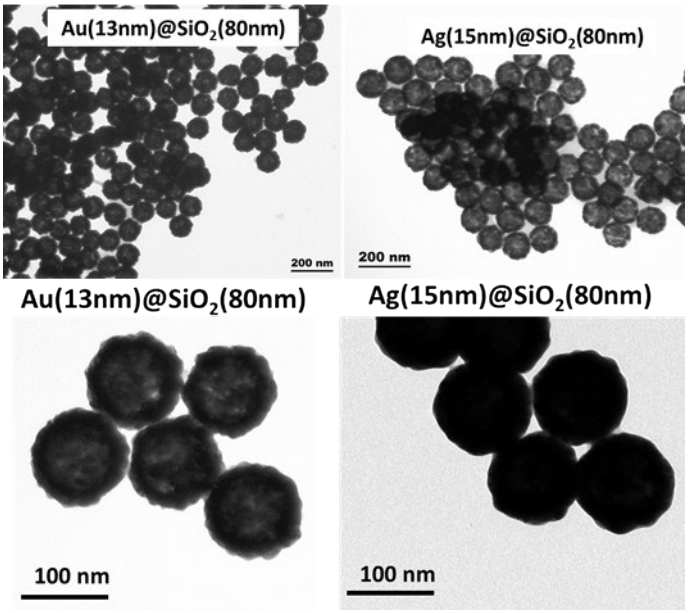

Figure SI-4. Gold and silver nanoshells fabricated through our process. Low magnification (top) conveys general aspect and homogeneity of the colloids. High magnification (bottom) shows the complete coverage of the silica cores by the metallic shells.

Hyperspectral Dark Field Microscopy.

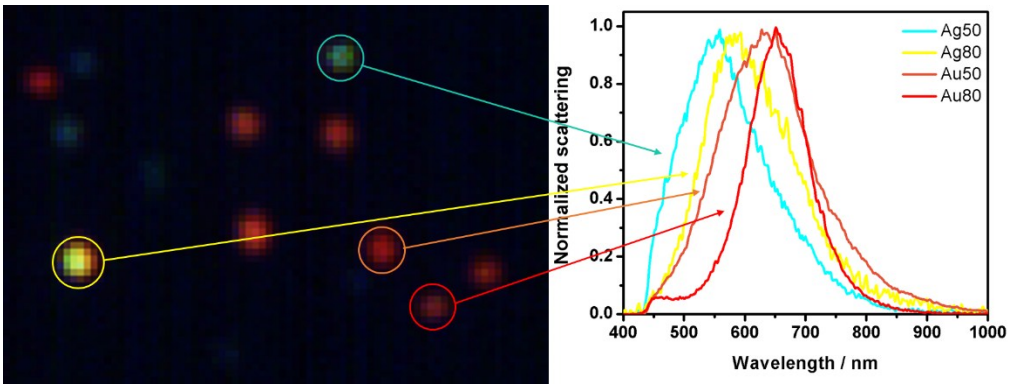

Figure SI-5. Hyperspectral image and scattering profiles of single nanoshells. Four samples were mixed and immobilized on an aminated glass coverslip. Hyperspectral image (left) shows isolated nanoshells and inserts pinpoint their respective scattering spectra (right).

Spectral separation and sample choice for dual labeling experiment.

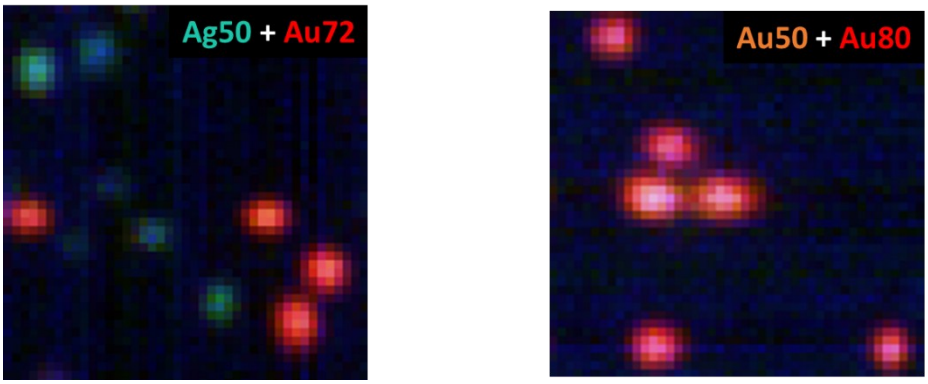

Figure SI-6. Hyperspectral analysis of combined particles for the dual-labeling experiment. It is clear the distinction between Ag50 and Au72 single nanoshells (left), while a good distinction between Au50 and Au80 nanoshells (right) can only be made with the use of hyperspectral analysis.

Hyperspectral analysis – Spectral variability

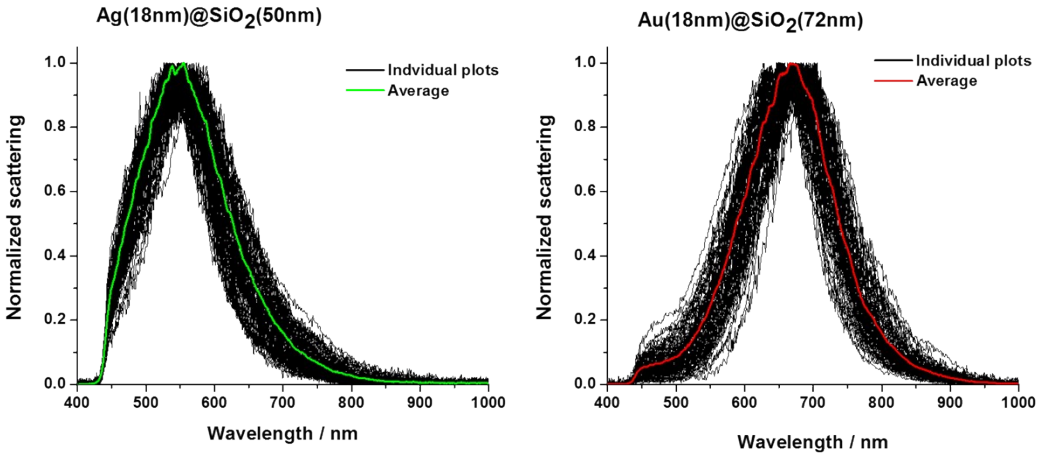

Figure SI-7. Individual extinctions from 100 nanoshells and the average extinction for Ag(18nm)@SiO¬2(50nm) and Au(18nm)@SiO2(72nm).

Spectral shift after bioconjugation.

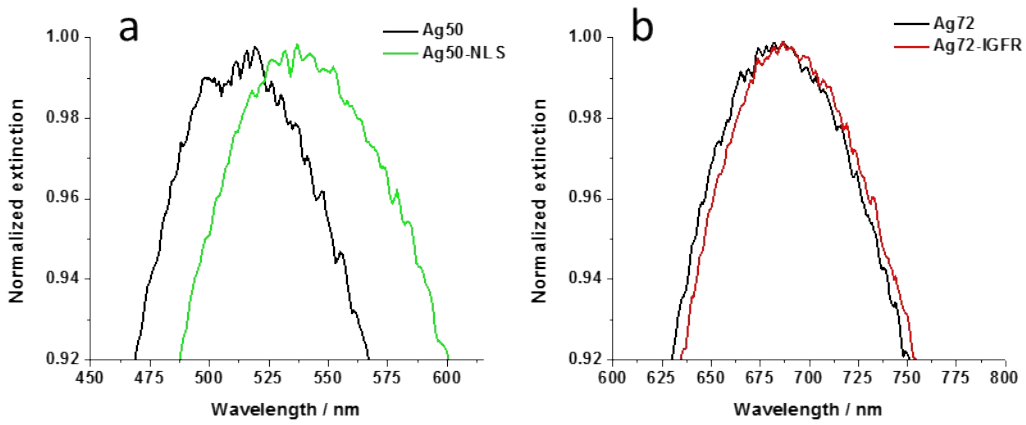

Figure SI-8. Spectral shift after bioconjugation. a) bioconjugation of the NLS peptide to the silver nanoshells, b) bioconjugation of the anti-IGFR antibody to the gold nanoshells.

The red shift in the LSPR of the colloids happen due to a change in refractive index on the surface of the nanoshells due to the attachment of the biomolecules.

Nonspecific endocytosis and nonspecific binding - controls for the cell labeling experiment.

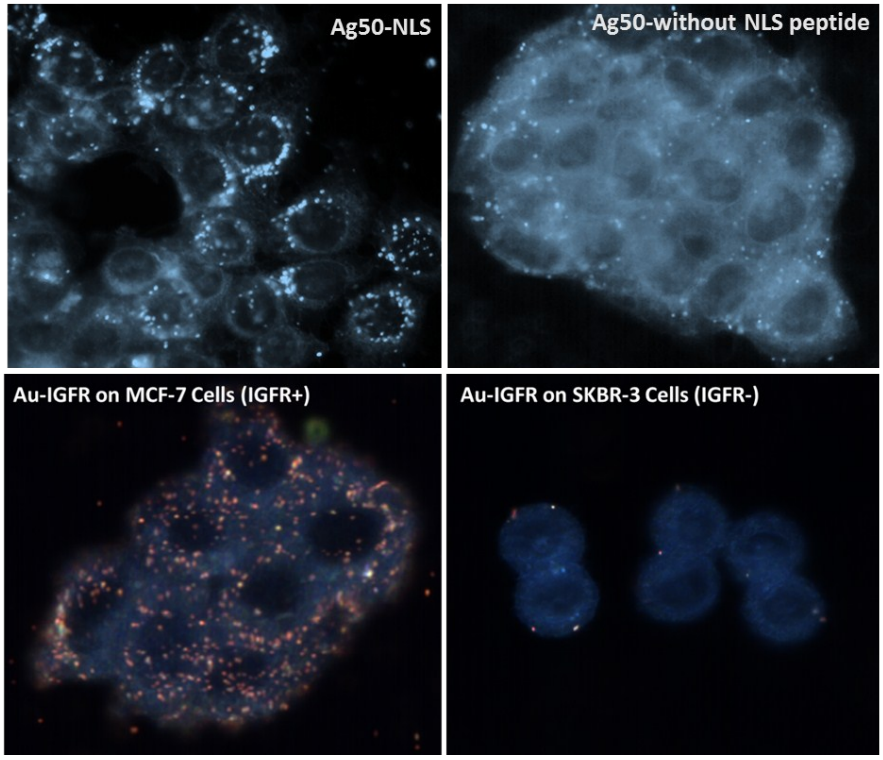

Figure SI-9. Negative controls. Hyperspectral images of Ag50-NLS (top left) and Ag50 without NLS peptide (top right); and Au-IGFR incubated with IGFR-positive MCF-7 cells (bottom left) and with IGFR-negative SKBR-3 cells (bottom right).

Silver nanoshells internalization and nuclear membrane labeling only happens in the presence of the NLS peptide. From SI-6 (top left), Ag-NLS nanoshells are easily visualized accumulating on the nuclear membrane, while in the absence of a NLS peptide, only a small number of particles are internalized and no selectivity for the nuclear membrane can be seen in Fig. SI-6 (top right). In the case of the anti-IGFR coated gold nanoshells (Au-IGFR), it's evident the accumulation on the plasma membrane of MCF-7 cells in Fig. SI-6 (bottom left), whereas only a few particles bind to the membrane IGFR-negative (basal levels of expression) SKBR-3 cells in SI-6 (bottom right). This control experiment proves the effective bioconjugation and point out to the excellent level of selectivity (for specific subcellular compartments) and specificity (for the IGF receptors on the plasma membrane) of this platform.

Labeling multiple compartments with gold nanoshells only

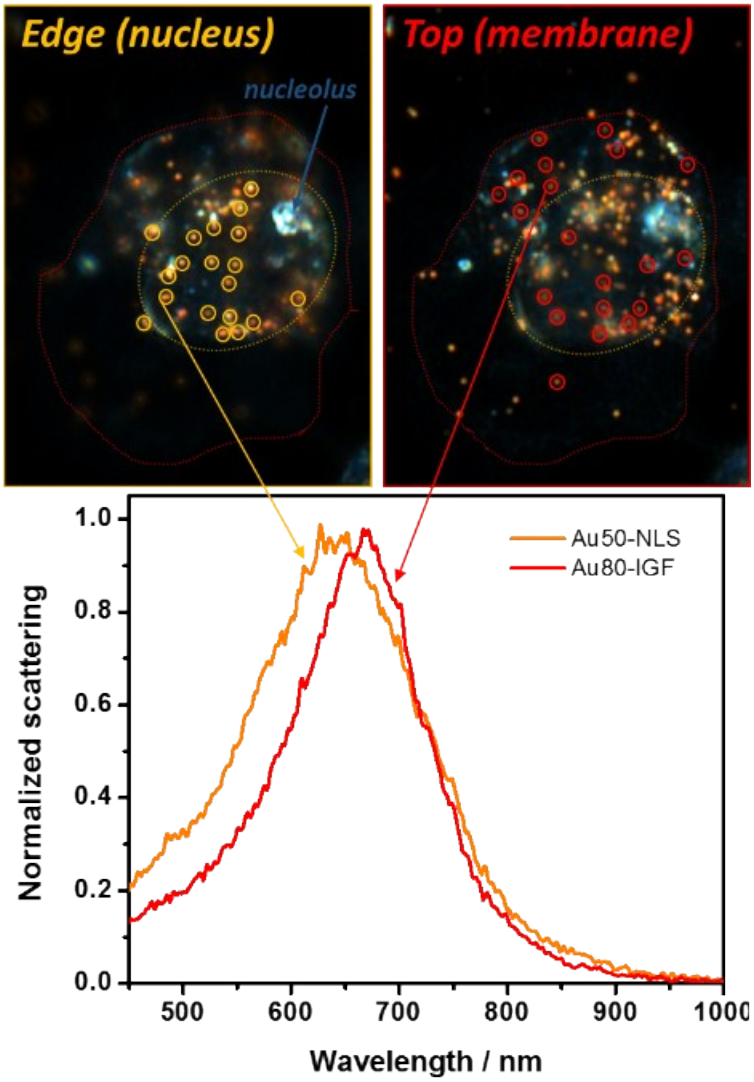

Figure SI-10. Dual-labeling with gold nanoshells only. Different focal depths show nucleus and membrane. Au50 nanoshells were coated with the NLS peptide and Au80 with the IGFR antibody. Due to the spectral proximity between the scattering profiles of Au50 and Au80, distinguishing between particles can only be achieved through hyperspectral microscopy. Hyperspectral sorting shows 20 nanoshells highlighted in orange (Au50) – left, and red (Au80) – right circles. Scattering spectrum for a single nanoshell is showed in the plot below the images. Nucleolus is shown by blue arrow.

Cell viability and fluorescence staining.

Cell viability 48 hours post-incubation was determined by the Trypan Blue exclusion method with the use of a hemocytometer. Table SI-2 summarizes the results.

Sample	Ag-NLS	Au-IGFR	Control (no nanoshells)
Cell viability (48h post-incubation)	90.8%	92.1%	91.4%

Cells were generally kept in growth media and washed 3 times with PBS in between incubations. In the case of propidium iodide staining, cells were incubated with Ag-NLS for 3 hours, washed and stained with 1 mL of a propidium iodide solution (1 μg/mL) for 10 minutes. They were then washed and mounted onto microscope slides for analysis. For Phalloidin CruzFluor® 488 staining we followed the manufacturer's protocol.

Imaging parameters.

All optical images displayed here were acquired using a Cytoviva Dual Mode Fluorescence/Hyperspectral Dark Field microscope. The imaging modes are easily interchangeable, facilitating comparative studies from the same visualization field. 60x and 100x objectives were used. While the setup is somewhat simple, they have an enclosed proprietary illumination system to which we can't gain access without damaging the setup. For detailed specifications we refer readers to the Cytoviva website (http://www.cytoviva.com). The light sources used in this work were a 150W Halogen lamp with Aluminum reflectors (Part # L1090 by Intl. Light Tech) with analog voltage control for dark field measurements, and an X-Cite® 120 Fluorescence Illumination System for the fluorescence measurements. Fluorescence and conventional Dark Field images were collected using a QImaging Retiga 4000 CCD. For the Hyperspectral measurements, the detector is switched to a Headwall spectrograph-coupled CCD.