Gold nanoparticles enhance fluorescence signals by flow

cytometry at low antibody concentrations.

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Supplementary material.

1. Materials and Methods.

1.1 Synthesis of the gold nanoparticles (AuNPs). The protocols applied to produce spherical gold nanoparticles (AuNPs) were previously described ^{1,2}. Briefly, AuNPs were synthesized using tetrachloroauric acid (HAuCl₄) and trisodium citrate ($C_6H_5Na_3O_7\cdot 2H_2O$) from Sigma-Aldrich (USA). The solution containing 50mL of ultra-pure water, HAuCl₄ 500µM and 5mL trisodium citrate 1% was mixed under ~ 80°C boiling and vigorous stirring until forming a ruby solution and left cooling down at room temperature, resulting in gold nanoparticles with an average size of about 20nm.

1.2 AuNP-Cetuximab bioconjugation. The protocol to produce Cetuximab-AuNP was

previously described ^{3,4}. Briefly, the isoelectric point of pH 8.5 was calibrated and 50µg of Cetuximab (5mg/mL) was added to 2mL pH adjusted AuNPs solution and incubated under continuous shaking at room temperature for 1h. Next, unbound Cetuximab was blocked by using BSA (Millipore, USA) at a final concentration of 1% (w/w), under continuous shaking at room temperature for 30min. Afterwards, the solution was centrifuged at 22.136 rcf for 20 min. The supernatant was disposed of, and the pellet was resuspended in sterile ultra-pure water.

1.3 UV-Vis absorbance. The extinction coefficient of each sample was measured in Visible and UV-Vis spectrophotometers Rayleigh Vis and Varioskan Flash spectral scanning multimode reader from 400nm to 800nm wavelengths (Thermo Scientific).

1.4 AuNP-Cetuximab concentration measurement. The quantification of cetuximab-AuNPs was carried out by absorbance at 280nm using a spectrophotometer NanoDrop 1000 (Thermo Fischer Scientific, USA)

1.5 DLS and Zeta potential measurements. Data were obtained using Litesizer 500 from Anton Paar, at room temperature in guintuplicate.

1.6 XPS measurements. Spectra were obtained using a SPECS spectrometer equipped with PHOIBUS 150 analyzer and conventional Mg K_{α} radiation (1253.6eV).

1.7 Fluorometric measurements. AuNPs were labelled with $BV421_{\lambda ex: 405mm, \lambda em: 421nm}$ (BD Horizont Brillant Violet 421) & PerCp-Cy 5.5 $_{\lambda ex: 490mm, \lambda em: 695nm}$ both from BD Biosciences (USA) and incubated under continuous shaking in the dark and at room temperature for 30 min. Samples were measured by the spectrophotometer Varioskan Flash spectral scanning multimode reader (Thermo Scientific).

1.8 Immunocytochemistry. 3×10^5 cells were seeded in a µ-dish imaging chamber (Costar, USA) for 24h. Next, cells were permeabilized using Triton-X 100/PBS/BSA (0.5% w/w) and were fixed using PFA 4% (Bio Rad, USA). Next, cells were treated with AuNPCet₅₀. Cells were

kept incubated for 1h in a humidified chamber at room temperature. Afterward, cells were labelled with Alexa Fluor 488[®] 5µg, from Molecular Probes and DAPI (2µg) from Sigma-Aldrich and incubated for 30min. The images were acquired using a fluorescence microscope Evos FL cell image system (Thermo Fischer Scientific, USA).

1.9 Cell cycle phases. $3 \ge 10^5$ cells were seeded in 6-well plates for 24h. A431 cells were exposed to 16µg of AuNPs and 16µg of AuNPCet 50µg for 1h. Next, cells were detached and stained with a home-made Hypochromic Fluorochrome Solution 0.5% containing Propidium lodide and Sodium Citrate dispersed in PBS 1X, for 30 minutes in the dark⁵. Data were acquired in a FACScanTM flow cytometer (BD Biosciences, USA) Ar-laser 488nm λ_{ex} and 564/606nm λ_{em} .

1.10 IFC image analysis. The specific plasma membrane stained with Alexa Fluor 488 was analyzed using the application Wizard in IDEAS[®] Software. The membrane region staining intensity was compared to the whole cell to validate the specific region marker.

1.11 Statistical analysis. Data were analyzed through the one-way ANOVA followed by Bonferroni correction as multiple hypothesis tests using the GraphPad Prism version 5. The mean and standard deviations were used with an interval of confidence of 95% and the value of p<0.05 was considered significant.

Supplementary figures.

1. Nanoprobe characterization size and nanoparticle concentrations.

Transmission electron microscope (TEM) images were analyzed using ImageJ software (NIH) and an average nanoparticles size of 20.1nm \pm 5.2nm was found. Both light scattering and Brownian motion were utilized to obtain nanoparticle diameter average of 43.0nm \pm 3.2nm. After bioconjugation with Cetuximab and labelling with Alexa Fluor 488 the average diameters were 73.4nm \pm 5.0nm and 651.8nm \pm 155.1nm, respectively, with a media PDI percentage of

27.0% ± 1.2%. Another sign of bioconjugation is the changes in charges of all samples, as shown in Supplementary Table 1. UV-Vis spectra suggest that a bioconjugation occurred as showed in Supplementary Figure 1 (a) and an indication of a successfully coated Cetuximab under concentration of 50µg is shown in Supplementary Figure 1 (b) which samples were measured according to Beer's law in 280nm. We also analysed the elemental composition of antibody conjugated AuNPs and mapped them by TEM where the presence of C covering the AuNPs were confirmed, as shown in Supplementary Figure 2 (a) with representative TEM images of the nanoprobe and panel (b) shows Carbon-K map and Carbon EELS measurements of the AuNPs functionalization, respectively.

Samples	TEM (nm)	DLS (nm)	PDI (%)	Zeta potential
				(mV)
AuNP	20.12 ± 5.21	43.03 ± 3.24	27.9 ± 0.7	-43.4 ± 0.9
AuNPCet 50µg		73.40 ± 5.00	28.0 ± 1.1	-35.5 ± 0.4
AuNPCet 50µg		651.85 ± 155.13	25.2 ± 2.6	-28.5 ± 0.3
+ Alexa 488				

Table S1: Gold	I nanoparticles	characterization
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Fig. S1: Nanoprobe characterization. Panel (a) shows UV-Vis spectra of bare AuNPs and conjugated AuNPs, respectively. Panel (b) shows AuNPCet_{50µg} concentration.



Fig. S2: AuNPs surface functionalization. Panels (a) and (b) shows AuNPs surface functionalization and panel (c) shows C map of their surfaces.

2. XPS analysis.

Photoelectron spectroscopy spectra were taken on a SPECS Instrument using Mg Ka (1253.6eV) radiation from an anode source operating at 120 W under a base pressure of 1×10^{-9} mbar. Samples were mounted on a Si wafer. Besides survey spectra shown in the main text, C1s, O1s, N1s, Na1s, and Au4f were taken separately and at the same experimental conditions of the electron analyzer (E_{pass} =20eV). For each sample, the charging effects during XPS measurement were corrected considering the main carbon peak (C1s) at 284.6eV as shown in Supplementary Figure 3 (a), and spectra have been fitted using Voigt line profiles. The estimated binding energies considering a tolerance of ±0.1eV are summarized in Supplementary Table 2.

Samples	01s	N1s	Na1s	Au4f _{5/2}	Au4f _{7/2}
NP-Au w/	530.5	399.2	1069.6	87.0	83.4
Cetux.					
NP-Au w/	530.5	399.2	1069.6	87.0	83.4
Cetux. +					
Alexa 488					
Percentage				(43%)	(57%)

Table S2: Binding Energies for O1s, N1s, Na1s, and Au4f, considering O1s at 284.6eV.

XPS survey spectra have shown (see Fig. 1(d)) weak Si 2s and Si2p lines, due to SiO_2 from the substrate, and that corresponding contribution for the O1s intensity was taken into account in the analysis of this line at 530.5eV- see Supplementary Figure 3 (b). The main N1s peak

shown in Supplementary Figure 3 (c) was found at 399.2eV, indicating unionized Nitrogen. For the Au-4f emission a comparison with the pristine Au-NPs is shown in Supplementary Figure 3 (d). For all samples, an electron binding energy of the Au4 $f_{7/2}$ transition at 83.4 eV was obtained, corresponding to a 0.6eV negative core level shift as compared to metallic bulk Au (84.0eV), fully consistent with nanoparticular Au⁰, preserved both in the sample with Cetuximab and after the Alexa 488 labeling. After functionalization with Cetuximab the intensity of the Au4f peaks is dramatically reduced due to the covering of the AuNPs. In order to estimate the atomic concentrations (as seen from XPS) the sensitivity factors of the photoemission lines were used in the calculations. Considering the stoichiometry of cetuximab $(C_{6485}H_{10042}N_{1732}O_{2023}S_{36})$, the theoretical concentrations related to Carbon are: O/C= 0.31, N/C=0.27 and S/C= 0.006. For Au_{NP}-Cet we have estimated O/C=0.38, and N/C=0.10. For AuNPCet+Alexa 488 the results were O/C=0.36, and N/C=0.09. The Sulphur signal in XPS is very week, almost under the detection limit, which is in line with Cetuximab bounded to AuNPs via sulphur termination. The Au/C values of 0.002 and 0.004 for AuNPCet and AuNPCet+Alexa 488, respectively, again indicate that both samples have been efficiently covered. Further comments on the elemental concentrations relative to Carbon for the Alexa 488 sample (NP-Au w/ Cetux.+Alexa 488) compared to the control sample (NP-Au w/ Cetux. Only) are as follows. Considering that the theoretical O/C for fluorescein ($C_{20}H_{12}O_5$) is 0.25 and for Cetuximab ($C_{6485}H_{10042}N_{1732}O_{2023}$ S₃₆) is 0.31, and that the Alexa Fluor 488 sample has attached fluorescein molecules, the Oxygen intensity is expected to slightly decrease, which is accordance with our findings. We have observed an increase of Na 30% of the Na-lines (Na1s and Na-Auger) after Alexa Fluor 488 labelling, which could be due to the excipient NaN₃ of Alexa Fluor 488.

In summary, from XPS, the elemental concentrations are in line with the coating of the surface-AuNPs with monoclonal antibody Cetuximab, as also observed in previous study⁴. After addition of Alexa Fluor 488, the modifications on the elemental concentrations, as detailed above, are consistent with the attachment of fluorescent-molecules to the NP_{Au}-Cetux.



Fig. S3: XPS spectra of C1s (a), O1s (b), N1s (c), and Au4f emission lines.

3. Fluorescence detection of AuNPs conjugated with fluorophores BV421 and PerCp-Cy 5.5.

AuNPs were coated with two different fluorophores BV421 and PerCp-Cy 5.5 to investigate whether a fluorescent nanoprobe could be formed and detected by fluorimetry. Both fluorophores were detected by this technique; however, data suggest that fluorimetry was more sensitive to detect AuNP-PerCp-Cy 5.5 than AuNP-BV421, as shown in Supplementary Figure 4.



Fig. S4: Fluorophore-gold nanoparticles binding. Image represents results from AuNPs coated with BV421 (a) and PerCp-Cy 5.5 (b). Samples were measured in triplicate (Mean \pm SD, * p<0.05).

4. A431 transmembrane EGFR binding.

The binding affinity of the nanoprobe was also assessed by fluorescence microscopy. The A431 cells were exposed to AuNPCet for 1h and labelled with Alexa Fluor 488 and DAPI. As A431 cells overexpress EGFR and Cetuximab is a monoclonal antibody to target this protein, the nanoprobe was efficiently bound to EGFR and detected by immunofluorescence as shown in Supplementary Figure 5.



Fig. S5: Representative immunocytochemistry image of A431 cells labelled with AuNPCet.

5. Gate strategy to select samples by flow cytometry.

To avoid doublets and dead (debris) cells during measurements, a gating strategy was delimited to establish a ROI for cell information analysis. Two strategies were made to assure the single cell analysis. For standard flow cytometry the SSC-A vs SSC-H approach was applied to gate single cells. Likewise, image flow cytometry allows determining a ROI for clusters of labelled nanoprobes, using the morphology approach. Aspect Ratio vs Area plot can gate single cells with high Aspect Ratio Values and lower the distribution rate in Area feature. The strategy used to determine single cell gates during acquiring and followed by the analysis of each sample is shown in Supplementary Figure 6.



Fig. S6: Gate strategy to select samples inside a ROI. Panel (a) shows A431 cell gate set measuring side scatter height (SSC-H) vs side scatter area (SCC-A) in conventional flow cytometry. Panel (b) shows A431 single cells selected by area for image coupled flow cytometry and panel (c) shows the region (R4) to detect clusters of labelled nanoprobes with Alexa Fluor 488.

6. Specificity of the nanoprobe labelling.

The constructed nanoprobe presented a high level of specificity due to its ability to be tagged efficiently by Alexa Fluor 488 anti-human. Neglectable unspecific binding was observed for anti-mouse secondary antibodies, meaning that our nanoprobe enhances fluorescence in a specific fashion, as shown in Supplementary Table 3.

Table

S3:

A431 fluorescence labelling.

Samples	Alexa Fluor	Alexa Fluor	Alexa Fluor
	488	555	647
Cetuximab	96.52 ± 4.91	0.21 ± 0.07	0.15 ± 0.03
AuNPCet 50µg	98.10 ± 1.20	0.22 ± 0.03	0.18 ± 0.04
Percentages	(% ± SD)	(% ±SD)	(% ± SD)

7. Image coupled flow cytometry fluorescence analysis.

In order to evaluate the immunocomplex specificity by image flow cytometry, the fluorescence signal in the entire cell region was measured using the morphology mask, that was designed to confirm the shape of the images (7a). The segmentation algorithm automatically defines the boundaries of each object, using the mask for calculating fluorophore intensity values. (Ideas Software, AMNIS, Merck Life Sciences). The fluorophore emission peak was observed under the membrane region, confirming the specificity of the immunocomplex AuNPCet Alexa Fluor 488 binding to EGFR in the A431 cell membrane (7b).



Fig. S7: Mask strategy used to measure the fluorophore emission by image region. Panel (a) shows the morphology mask boundaries. Panel (b) shows the fluorophore emission peak in the membrane region (red lines).

8. Cell cycle analysis of A431 cells.

To ensure the conditions of A431 cells during the experiments they were exposed to AuNPs and AuNPCet and their cell cycle phases were assessed. Under experimental conditions Gap 1 region (G1) was above 50% indicating that the biosynthetic activities of the cells were in course. It was also noticeable that DNA synthesis phase (S) stayed around 30% indicating chromosomal replication. All samples presented Gap 2 region (G2) percentages below 10% indicating that cells can go through mitosis. Altogether these data show the same cell cycle pattern of all samples meaning that no bias was found regarding the quality of the cells during experimental procedures, as shown in Supplementary Figure 8. The percentage of each cell cycle phase evaluated is shown in Supplementary Table 4.



Fig. S8: Cell cycle phase analysis. Cells were stained with HFS 0.5% and each profile is demonstrated in panel (a). Panel (b) shows no difference among evaluated samples.

Phase (G1)	Phase (S)	Phase (G2)
54.04 ± 1.35	36.12 ± 1.43	7.26 ± 0.38
57.46 ± 2.21	32.24 ± 1.22	8.16 ± 1.67
57.98 ± 0.92	30.82 ± 0.83	9.05 ± 0.19
(% ± SD)	(% ±SD)	(% ± SD)
	Phase (G1) 54.04 ± 1.35 57.46 ± 2.21 57.98 ± 0.92 (% ± SD)	Phase (G1) Phase (S) 54.04 ± 1.35 36.12 ± 1.43 57.46 ± 2.21 32.24 ± 1.22 57.98 ± 0.92 30.82 ± 0.83 (% ± SD) (% ±SD)

Table S4: A431 cell cycle phase percentage.

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References

- J. Turkevich, P. Stevenson Cooper and J. Hillier, *Discuss. Faraday Soc.*, 1951, **11**, 55–75.
- 2 G. Frens, *Nat. Phys. Sci.*, 1973, **241**, 20–22.
- 3 Y. Qian, M. Qiu, Q. Wu, Y. Tian, Y. Zhang, N. Gu, S. Li and L. Xu, *Sci. Reports*, 2014, 15, 1–8.

- L. M. Andrade, E. M. N. Martins, A. F. Versiani, D. S. Reis, G. Flavio, I. P. De Souza,
 R. M. Paniago, E. Pereira-maia and L. O. Ladeira, *Mater. Sci. Eng. C*, 2020, 107, 110203.
- L. M. Andrade, J. M. Geraldo, O. X. Gonçalves, M. T. T. Leite, A. M. Catarina, M. M. Guimarães, A. F. P. Leme, S. Yokoo, C. R. Machado, M. A. Rajão, S. M. Carvalho, D. A. Gomes, C. J. Aguiar, E. M. Souza-Fagundes, C. L. Zani, R. R. Resende, O. A. Martins-Filho and M. F. Leite, *J. Cancer Sci. Ther.*, DOI:10.4172/1948-5956.1000127.