SUPPLEMENTARY MATERIAL

Real matrix-matched standards for quantitative bioimaging of cytosolic proteins in individual cells using metal nanoclusters as immunoprobes-label: a case study using laser ablation ICP-MS detection

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Abstract

This Supplementary Material contains some details related to the Experimental Section, including Reagents, Experimental Methods and Instrumentation. Concerning the Results and Discussion section, different Figures and Tables are included showing experimental results related to optimizations of the immunocytochemistry assay, the analysis of HRPEsv cells by LA-ICP-MS and the characterisation of HRPEsv cells@AuNCs standards.

EXPERIMENTAL SECTION

A. Reagents

A.1. Synthesis of monodisperse AuNCs.- $AuCl_4Na \cdot H_2O$ (99% powder; Sigma-Aldrich), lipoic acid (>98% powder; Across Organics) and $NaBH_4$ (98% powder, Sigma Aldrich) were the reagents required to obtain monodisperse water dispersible AuNCs. Moreover, NaOH (Sigma-Aldrich) and propan-2-ol (Fisher Scientific) were used during the protocol.

A.2. Synthesis and characterization of AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A immunoprobes.- The carbodiimide strategy was chosen to carry out the bioconjugation of AuNCs with the specific antibodies. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (98% powder; Across Organics) and the N-hydroxysuccinimide (NHS) (> 98% powder; Sigma-Aldrich) reagents were used for this purpose. Goat polyclonal anti-human Apolipoprotein E (anti-h-APOE) (Fisher Scientific) and rabbit polyclonal anti-human Metallothionein 2A (anti-h-MT2A) (Cloud Clone Corporation) were the specific primary antibodies selected. Amicon ultra centrifugal filter units (3 and 100 kDa pore size, Merck Millipore) were employed for the purification of the AuNCs and the AuNCs-immunoprobes. Regarding the characterization of the immunoprobes (AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A), a non-competitive direct ELISA immunoassay was performed to know the available antibody. For such purpose protein IgG standard from mouse (University of Oviedo), secondary antibody labelled with peroxidase (HRP) against mouse proteins (Sigma-Aldrich), the TMB-$H_2 0_2$ substrate (Sigma-Aldrich), and sulphuric acid (95-97%, Merck Millipore) were used. General reagents such as deionized ultrapure water, resistivity 18.2 MΩ·cm, (Purelab Flex 3&4; ELGA-Veolia) was used throughout as well as HNO_3 (67-69%, trace analysis; VWR chemicals) to prepare solutions for ICP-MS measurements. In addition, hydrochloric acid (HCl 37%, VWR) was used together with HNO_3 to produce aqua regia (3HCl:HNO_3) for the digestion of the AuNCs.

A.3. Human RPE cell line.- The cell line used in this work is the human immortalized RPEsv40 (HRPEsv), which is well stablished in our laboratory [1] During cell growth and expansion, Dulbecco´s modified eagle medium/nutrient mixture F-12 (DMEMF12) (Invitrogen), supplemented with 1% (v/v) penicillin/streptomycin (P/S) and 10% (v/v) fetal bovine serum (FBS) (both from Gibco™, Thermo Fisher Scientific) was used. Additionally, CD hybridoma free-serum medium (Gibco™, Thermo Fisher Scientific), supplemented with L-glutamine (Thermo Fisher Scientific) and P/S, was used with the cells submitted to the immunocytochemistry (ICC) for determination of

the target proteins. Moreover, other reagents were used for the cell preparation, such as recombinant human interleukin-1α (IL1α) from Gold Bio for the pro-inflammatory treatment of the cells, 0.25% trypsin-EDTA (Gibco™, Thermo Fisher Scientific) employing for collecting the cells from the seeding flasks, and 4% paraformaldehyde (PFA, VWR chemicals) to perform cell fixation. All materials and reagents were sterilized or/and autoclaved to avoid any contaminations. HRPEsv cells used for the ICC using AuNCs-immunoprobes (fluorescence and LA-ICP-MS analysis) were grown directly on slides with the Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Fisher Scientific), denoted as chamber slides.

A.4. Immunocytochemistry assay.- Regardless the detection employed (fluorescence or MS), several reagents were required for the ICC, including triton X-100 (Sigma-Aldrich) for cell membrane permeabilisation and bovine serum albumin (BSA) (99% powder, Merck Millipore) together with donkey serum (Sigma-Aldrich) to block non-specific interactions. All the washing steps were performed with phosphate-buffered saline (10 mM PBS, pH 7.4) (Sigma Aldrich) with a percentage of surfactant tween 20 (Sigma-Aldrich).

Two types of measurements were carried out by fluorescence: (i) Direct detection using AuNCs-immunoprobe (AuNCs:anti-h-APOE or AuNCs:anti-h-MT2A), where AuNCs fluorescence was measured, and (ii) Indirect detection following the emission of fluorophore Alexa®. In this last case it should be noted that primary antibodies without AuNCs were used: Alexa® Fluor Plus (A32731 and A32814), consisting on Alexa® 594:goat antirabbit IgG and Alexa® 488:donkey antigoat IgG as secondary antibodies for detecting anti-h-MT2A and anti-h-APOE, respectively. Moreover, 4′6-Diamidino-2- phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific) was employed to stain the nucleus cell in fluorescence detection.

A.5. Study of the MT2A and APOE gene expression by quantitative real time polymerase chain reaction (qRT-PCR).- In order to understand the gene expression of the MT2A and APOE proteins in HRPEsv cells (both in CT cells and cells treated with IL1α), a quantitative real time polymerase chain reaction (qRT-PCR) was assayed. For such purpose, HRPEsv cells were trypsinized and the total RNA content was isolated using the RNeasy Mini Kit (Quiagen, USA). RNA quality and concentration was analyzed using PicodropTM spectrophotometer (Picodrop Limited). From 1 µg of total RNA, complementary DNA (cDNA) was obtained using reverse transcriptase enzyme ("High Capacity RNA-to-cDNA", Applied Biosystems^{TM}). Gene expression of MT2A (Hs02379661_g1) and APOE (Hs00171168_m1) was determined on a 7500 RT-PCR system

employing the TaqMan® probe (Applied Biosystems, Thermo Fisher). Glucuronidase gene (GUSB; Hs00939627_m1) was used as an endogenous control and each experiment was performed in triplicate.

A.6. Quantitative ELISA commercial kits for MT2A and APOE determination.- Two commercial enzyme-linked immunosorbent assay kits from Cloud-Clone Corporation were employed for the determination of MT2A (SEB868Hu 96 Tests) and APOE (SEA704Hu 96 Tests) proteins concentration in the cell lysates from HRPEsv cell cultures (both in CT and IL1α–treated cells). Furthermore, the buffer Tris-HCl (pH 7.4) (Sigma Aldrich) was required to resuspend the cellular pellet just before performing the lysis of the cells using the ultrasound probe to extract the cytosolic content.

B. Experimental methods

B.1. Synthesis and characterization of AuNCs and AuNCs labelled immunoprobes.- The experimental procedure for the synthesis of AuNCs has been previously described [2] The just-synthesized AuNCs (size-dispersed AuNCs) were subjected to a post-treatment in order to achieve size focusing, resulting in a decrease of the deviation associated with their sizes. To this end, AuNCs were heated at 50ºC for 2 h in the same flask where they were initially synthesized. Briefly, the synthesis protocol is as follows: a water solution of lipoic acid (0.035 g in 20 mL of water) with a pH of 8 (adjusted by adding 0.050 mL of 2 M NaOH) was prepared in a cylindrical flask where 200 µL of 50 mM $NaAuCl_4$ were subsequently added. After mixing properly with a magnet, 400 µL of 50 mM of $NaBH_4$ (in propanol) were dropwise added with a syringe into the flask containing the metal precursor and the capping ligand water solution. After 4 h at RT under continuous stirring, the AuNCs just synthesized (disperse) were then subjected to a post-treatment of aging (50ºC for 2 h) for obtaining monodisperse NCs (i.e., size-focusing of disperse AuNCs). The average diameter obtained for the AuNCs (measured by high resolution transmission electron microscopy, HR-TEM) was 1.99 ± 0.04 nm. Afterwards, AuNCs were purified by ultracentrifugation and characterized by ICP-MS, HR-TEM and fluorescence, following a protocol similar to that previously reported for the synthesis of disperse AuNCs [3].

Monodisperse AuNCs were independently bioconjugated to the specific antibodies (anti-h-APOE or anti-h-MT2A) following the carbodiimide method [4]. For such purpose, 100 µL of 100 µg mL^{-1} of each primary antibody were mixed with an excess of AuNCs (1:3 molar ratio of antibody:AuNCs) to ensure complete labelling. Subsequently, the reagents necessary to carry out the amide bonding

were also added in excess (1:1200:1:200 molar ratios of antibody:EDC:NHS) to the solution and it was being shacked for 2 h using the vortex. After purification by ultracentrifugation, the just-synthesized AuNCs-immunoprobes were stored into the refrigerator at 4ºC until further use. For the complete characterization of the AuNCs-immunoprobe (required to be used as specific label for the quantitative analysis of proteins by LA-ICP-MS), quantification of the available antibody as well as the total amount of Au in the immunoprobe must be known. Thus, to characterize AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A immunoprobes a similar procedure to that previously described by Cruz-Alonso *et al.* [5] was followed. Briefly, two types of experiments were required: a direct ELISA with an IgG protein as the calibration standard (concentrations from 0 to 1 mg mL^{-1}) to determine the available concentration of antibody in the AuNCs-immunoprobe and ICP-MS analysis to determine the Au concentration in the immunoprobe. In this way, it was possible to determine the stoichiometry of the AuNCs-immunoprobe.

B.2. Culture and treatment of HRPEsv cells.- For qPCR assay as well as the preparation of HRPEsv cells@AuNCs standards, 6-well-plate supports were selected. In this case, around $1 \cdot 10^6$ cells/well were seeded for each condition (CT and IL1α for qPCR) and for each AuNCs concentration (HRPEsv cells@AuNCs standards). In the case of ELISA assays, a higher cells concentration was necessary: HRPEsv cells in the range of $10 \cdot 10^6$-$20 \cdot 10^6$ cells per condition were required (T150 flasks were used to seed the cells).

B.3. Preparation of "single-cell laboratory standard": HRPEsv cells@AuNCs.- In order to perform a matrix-matched calibration by LA-ICP-MS, HRPEsv cells were supplemented with different concentrations of AuNCs (0, 5, 25, 50, 100, 200 µg mL^{-1} in DMEMF12 for 24 h). Conventional nebulization ICP-MS analysis was carried out to determine the Au concentration in HRPEsv cells@AuNCs. For such purpose, HRPEsv cells @AuNCs were collected from the 6-well-plates: cells were incubated with 0.5 mL/well of 0.25% of Trypsin-EDA (pH 7.4) inside the incubator during 3 min. Then, 2 mL of complete medium DMEMF12 (+10% FBSi +1% of P/S) was added to block trypsin activity. After collecting the cellular pellet by centrifugation and washing twice with PBS, cells were manually counted with the haemocytometer. Subsequently, cellular digestion was carried out using aqua regia [6]; 0.5 mL of aqua regia (HNO_3:3HCl) was added per cellular pellet and after 1 h, they were under ultrasound vibration for 2 h. HRPEsv cells@AuNCs digestions were finally diluted with ultrapure water, until an adequate concentration, for ICP-MS measurements. An external calibration with liquid Au standards, using Pt as internal standard, was

performed by ICP-MS to determine the average Au concentration uptake per cell, for the different concentrations of AuNCs. Experimental conditions for the determination of Au in HRPEsv cells@AuNCs standards are shown in **Table S1**.

Table S1. Operating conditions for the analysis of HRPEsv cells@AuNCs by conventional nebulization ICP-MS and the analysis of CT and IL1α–treated HRPEsv cells by LA-ICP-MS.

Parameter (7900 Agilent ICP-MS)	**Value**
ICP RF power (W)	1500
Plasma gas flow (mL min^{-1})	18
Nebulizer gas flow (mL min^{-1})	0.9 (ICP-MS) or 0.75 (LA-ICP-MS)
Isotopes (m/z)	^{197}Au, ^{195}Pt (IS)
Dwell time (ms)	100 (ICP-MS) or 50 (LA-ICP-MS)
Parameter (NWR193 ESI - LA)	**Value**
Laser diameter, spot (μm)	6
Scan speed (μm s^{-1})	5
Fluence (J cm$^{2\ -1}$)	0.3 (5%)
Frequency (Hz)	20

B.4. Determination of APOE and MT2A proteins in HRPEsv Cells by ELISA.- HRPEsv cells analysed using the commercial ELISA kits were trypsinized by incubating 6 mL per flask of 0.25% trypsin-EDTA for 3 min (37°C in 5% CO_2). Later, 20 mL per flask of DMENF12 were added to stop the trypsin activity and the cells were collected into different falcon tubes to obtain the cellular pellet from the supernatant by mild centrifugation (3 min at 1176 g). Finally, to remove dead cells that remain in the medium, cells were washed once with PBS and after spinning-off (3 min at 1176 g), cellular pellets were collected. Afterwards, 500 μL of Tris-HCl buffer (pH 7.4) was added to resuspend the cellular pellet just before performing the cellular lysis using the ultrasound probe with the objective of extracting the cytosolic content. For such purpose cellular pellets were ultrasound 10 KHz intermittently for 30 s (3 times) on iced bath. The cell lysate was then centrifuged (15300 g x 30 min at 4°C). Eventually, the supernatant (cytosolic fraction) was collected and stored at -80 °C for APOE and MT2A quantification with the ELISA kits meanwhile the pellet (cell membrane) was discarded.

B.5. Study of the MT2A and APOE gene expression by qRT-PCR.- Cellular pellets ($\sim 1 \cdot 10^6$ cells) were collected from the growth flaks following the Kit specifications and using the buffers and reagents providing by the commercial kit. Cellular pellets from CT and HRPEsv cells after the pro-inflammatory treatment with IL1α (3 replicates per condition) were firstly trypsinized and the total RNA content was isolated following *RNeasy Mini Kit* instructions. Then, RNA concentration was determined by Picodrop™ spectrophotometer, using a known concentration of BSA as calibration standard, and measuring the absorbance ratio of 260/280 nm. From 1 µg of RNA, complementary DNA (cDNA) was obtained using reverse transcriptase enzyme ("High Capacity RNA-to-cDNA") using the following temperature conditions:5 min at 25 ºC, 90 min at 37ºC and then 5 min at 95ºC. Once cDNA was obtained, gene expression of APOE and MT2A was determined using the fluorophore TaqMan® probe by fluorescence detection. All PCR amplifications were performed with the thermal cycling conditions of 95ºC for 10 min, followed by 40 cycles of 95ºC for 15s and 60ºC for 1 min The PCR cycles were compared with an endogenous gene control which was in our case the gen GUS (experiments were performed in triplicate). The samples were kept in ice and all the material and reagents used were free-RNAsas.

C. Instrumentation

For cell counting, a Neubauer haemocytometer plate (Sigma Aldrich) was used under an optical microscope (Inverted Microscope Leica DM IL LED, Leica Microsystems) at 10-fold magnification. ELISA measurements were carried out with the spectrophotometer 2030 Multilabel reader VICTORTM X5 (PerkinElmer). Regarding the gene expression, PicodropTM fluorimeter (Thermo Fisher) was used to quantify RNA per cell lysate aliquot. Thermocycler 96 wells Veriti™ HID as well as 7500 RT-PCR system (both from Applied Biosystems™, Thermo Fisher) were employed to carry out the RNA to cDNA transcription and quantitative PCR, respectively.

RESULTS AND DISCUSSION

Optimizations of the immunocytochemistry assay

AuNCs:immunoprobe characterization.- For absolute protein quantification, the average number of Au atoms per immunoprobe (i.e., the amplification factor) must be known. Two types of experiments were carried out after performing the bioconjugation procedure (AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A) following a similar protocol to that described by Pereiro *et al.* [5,7] The quantification of the available antibody after the bioconjugation was determined with a direct ELISA using IgG protein standard to build the calibration curve (concentrations from 0 to 1 mg mL^{-1}). Experimental results showed a concentration for the available antibody of 0.074 ± 0.003 µg mL^{-1} ($2.95 \cdot 10^{15} \pm 0.12 \cdot 10^{15}$ moles). Concerning the total amount of Au in the AuNCs-immunoprobes, digested bioconjugates were analysed by conventional nebulization ICP-MS and the moles of atoms of Au were $1.37 \cdot 10^{18} \pm 0.23 \cdot 10^{18}$. Thus, the amplification factor calculated as the ratio between the number of Au atoms per immunoprobe and the moles of available antibody was found to be 466 on average (466 ± 18).

Optimization of the primary antibodies concentration.- To ensure a complete recognition of the target proteins (APOE and MT2A) in HRPEsv cells by the AuNCs-immunoprobe, the concentration of the primary antibodies in the specific tag was studied. Following the dilution range recommended for the antibodies specifications, three serial dilutions of the antibodies stock concentration were independently assayed in CT and IL1α−treated HRPEsv cells. The stock concentrations of anti-h-APOE and anti-h-MT2A were 1 mg mL^{-1} and 0.23 mg mL^{-1}, respectively. An indirect fluoroimmunoassay using Alexa® (secondary antibody fluorescent tag), was carried out testing three different dilutions for each primary antibody (unconjugated with AuNCs). As an example, **Fig. S1 (a, b, c and d)** shows the fluorescence images obtained for the APOE distribution in HRPEsv cells following the green colour of Alexa®488 for a negative control sample (without the primary antibody) and 1:50, 1:100 and 1:200 dilutions, respectively. It can be observed that the intensity was kept constant for 1:50 and 1:100 dilutions. However, the intensity was significantly lower with 1:200 (**Fig. S1d**), indicating that the highest dilution tested (1:200) was not enough to completely detect APOE content in HRPEsv cells. Concerning 1:50 and 1:100 dilutions, the 1:100 dilution (10 µg mL^{-1}) of the primary antibody was selected for the AuNCs:anti-h-APOE immunoprobe (signals in the same range than those obtained for 1:50). Concerning dilution between

1:100 and 1:200, it should be noted that washing steps carried out during the ICC assay allowed to remove the non-labelled antibody.

A similar study was performed for MT2A protein. **Fig. S1 (e, f, g, h)** shows the fluorescence images obtained for MT2A distribution in HRPEsv cells (red colour due to Alexa®594) for a negative control sample (without the primary antibody) and 1:23, 1:46 and 1:115 dilutions of the antibody, respectively. In this case, a 1:46 (5 µg mL^{-1}) was selected as the adequate dilution of the primary antibody for performing the total detection of MT2A protein in HRPEsv cells. 1:115 dilution (2 µg mL^{-1}) was discarded because of the low fluorescence intensity, and 1:23 and 1:46 dilutions exhibited similar fluorescence signals. Noted that **Fig. S1a** and **S1e** are the negative controls of the immunoassay for APOE and MT2A, respectively. In this case, those images enabled to discard any nonspecific detection by the secondary antibodies (Alexa® 488:donkey antigoat IgG and Alexa® 594:goat antirabbit IgG), demonstrating that the intensity shown in fluorescence images only corresponded to the detection of the target proteins.

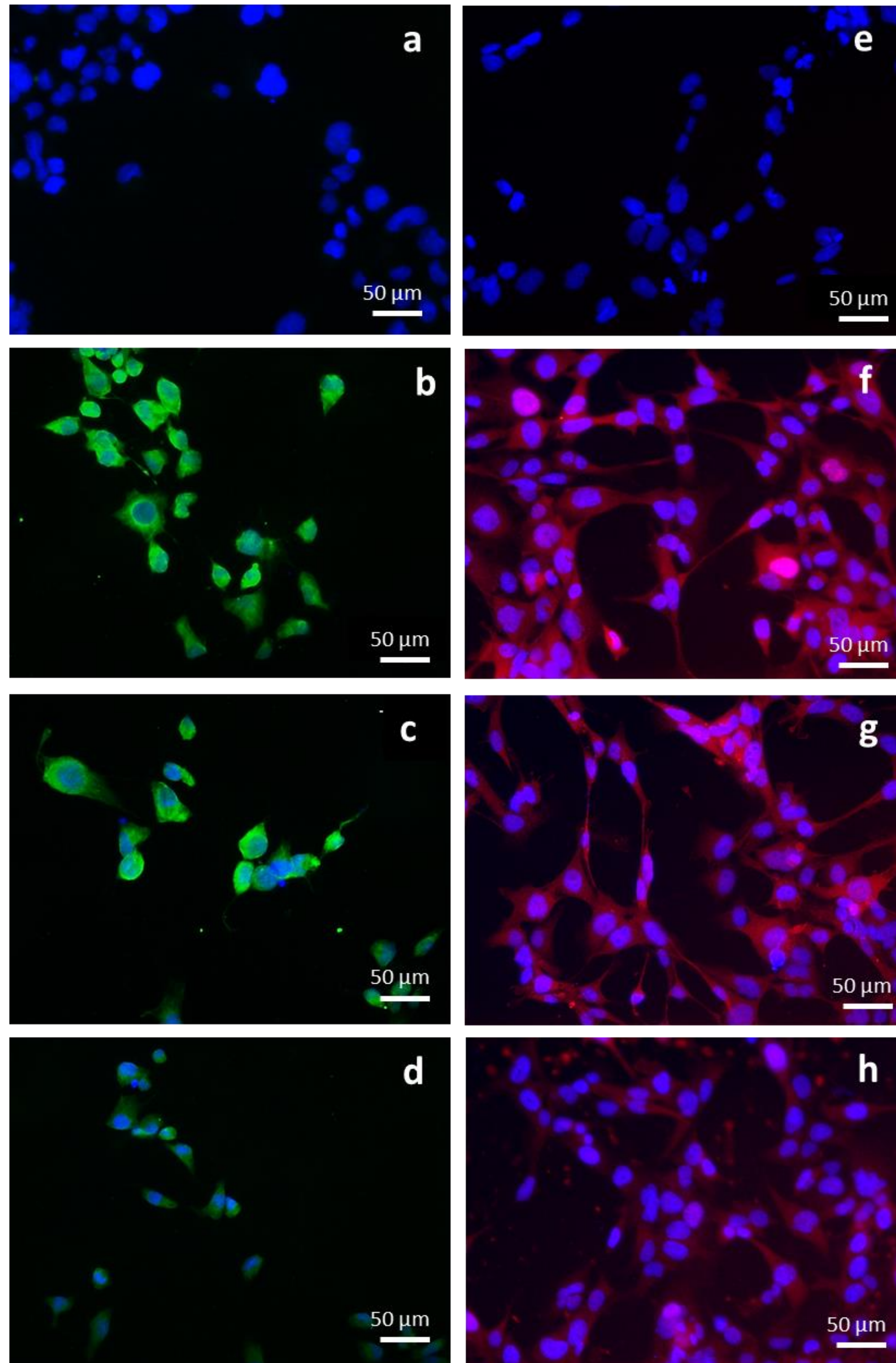

Figure S1. Fluorescence images obtained for the indirect fluoroimmunoassay using Fluor Alexa® for the optimization of the primary antibodies concentration for the detection of APOE and MT2A proteins in HRPEsv cells. a) Anti-h-APOE, negative control (w/o primary antibody), b) 1:50 dilution anti-h-APOE, c) 1:100 dilution anti-h-APOE, d) 1:200 dilution anti-h-APOE, e) Anti-h-MT2A, negative control (w/o primary antibody), f) 1:23 dilution anti-h-MT2A, g) 1:46 dilution anti-h-MT2A, and h) 1:115 dilution anti-h-MT2A. Green colour in APOE images is due to the Fluor Alexa® 488 revealed, whereas red colour in MT2A images is due to Fluor Alexa® 594.

Identification of cytosolic proteins in individual HRPEsv cells by LA-ICP-MS

Fig. S2 shows the histogram obtained for the analysis of APOE protein in two CT and two IL1α–treated HRPEsv cells (2D-images of the cells are in **Fig. 2**), representing the number of pixels for each $^{197}Au^{+}$ intensity signal. **Fig. S3** collect the fluorescence images obtained by laser confocal microscopy for APOE distribution in individual HRPEsv cells using direct (AuNCs-immunoprobe) and indirect (secondary antibody with fluorophore Fluor Alexa®488) fluorescence detection. On the other hand, **Fig. S4** shows the fluorescence image obtained for AuNCs negative controls in CT and IL1α–treated cells, demonstrating the absence of unspecific interactions. Regarding MT2A protein, **Fig. S5** depicts the histogram obtained for several CT and IL1α–treated cells (2D-images of the cells are in **Fig. 2**), representing the number of pixels for each $^{197}Au^{+}$ intensity signal.

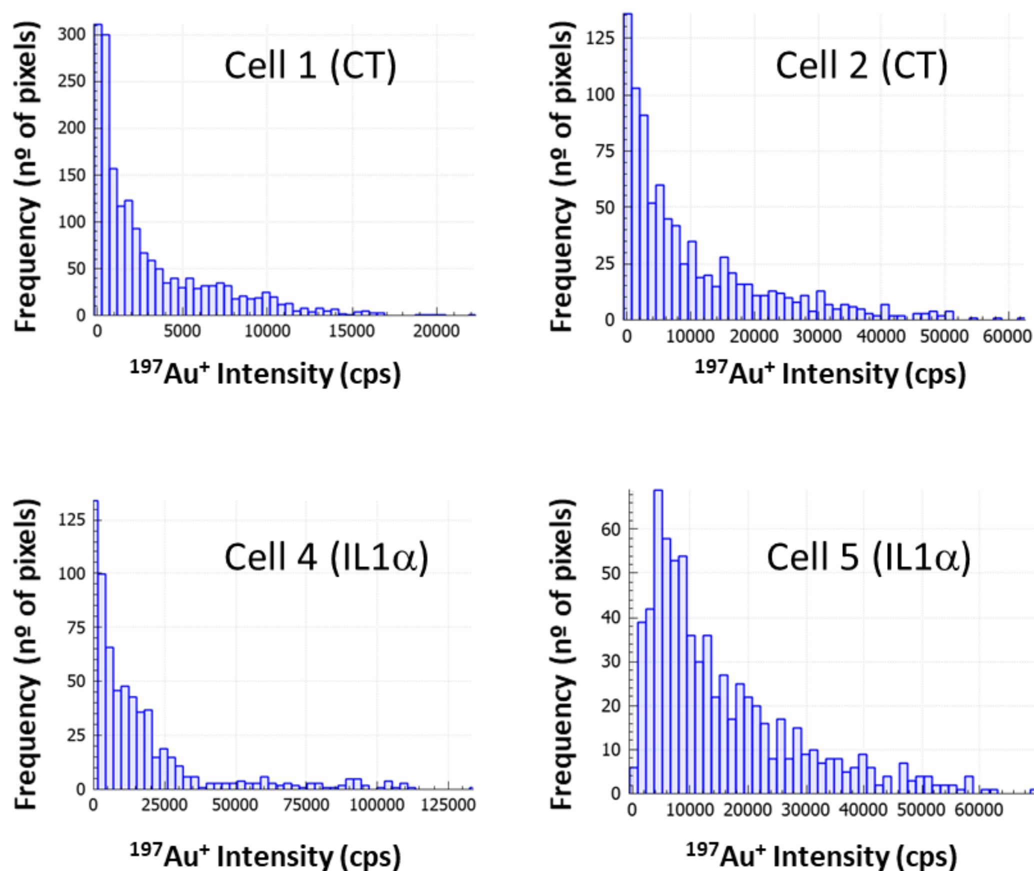

Figure S2. Histograms of pixel frequency per $^{197}Au^{+}$ intensity signal obtained for the analysis of APOE in CT (cell 1 and cell 2) and IL1α–treated (cell 4 and cell 5) HRPEsv cells analysed by LA-ICP-MS. AuNCs:anti-h-APOE immunoprobe was used in ICC for detection of APOE protein.

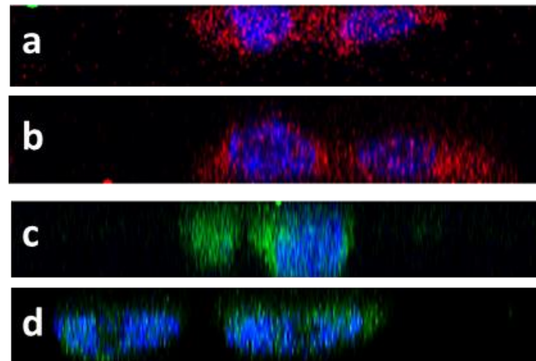

Figure S3. Confocal in-depth (x-z) images obtained for APOE distribution in individual HRPEsv cells. a) CT cells; DAPI (blue) and AuNCs:anti-h-APOE immunoprobe (APOE distribution in red), b) IL1α−treated cells; DAPI and AuNCs:anti-h-APOE immunoprobe, c) CT cells; DAPI (blue) and secondary antibody Fluor Alexa®488 (APOE distribution in green), and d) IL1α−treated cells; DAPI and secondary antibody Fluor Alexa®488.

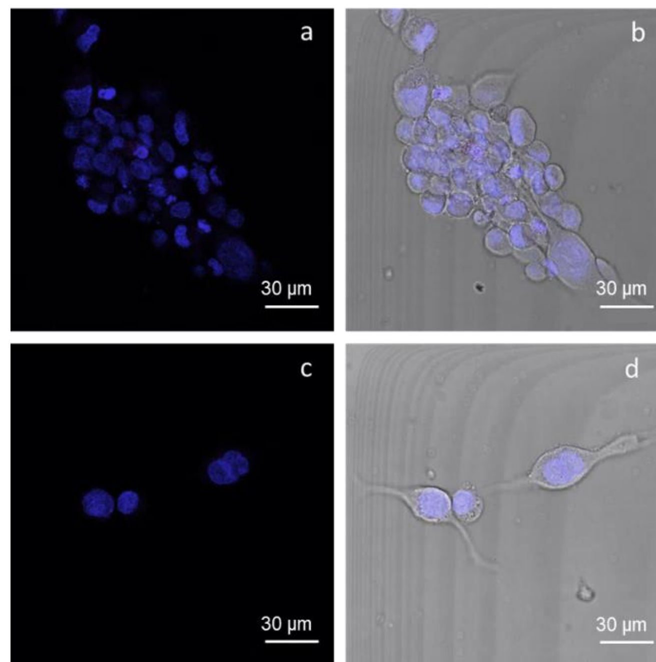

Figure S4. Fluorescence images obtained by laser confocal microscopy from negative controls of the immunosassay employing AuNCs as fluorescence tag; free AuNCs (i.e., unconjugated) was added to HRPEsv cells to check the presence of unspecific interactions of AuNCs and cells. a & b) CT HRPEsv cells, and c &d) IL1α−HRPEsv cells.

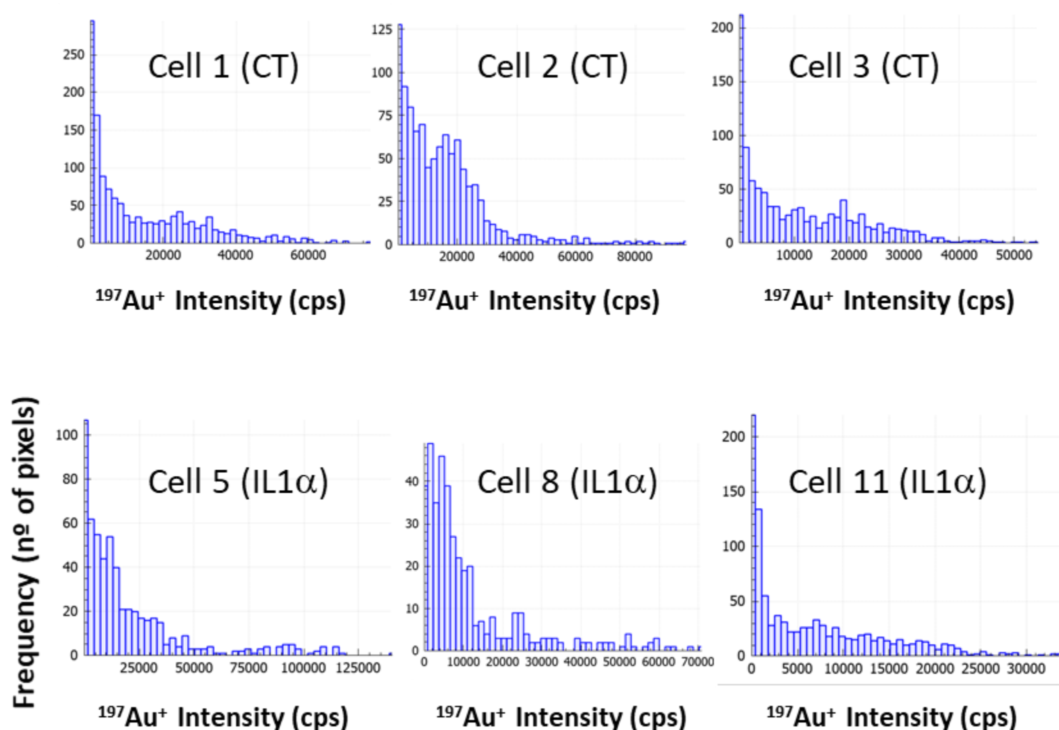

Figure S5. Histograms of pixel frequency per $^{197}Au^{+}$ intensity signal obtained for the analysis of MT2A in CT (cells 1, 2 and 3) and IL1α–treated (cells 5, 8 and 11) HRPEsv cells analysed by LA-ICP-MS. AuNCs:anti-h-MT2A immunoprobe was used in ICC for detection of MT2A protein.

HRPEsv cells supplemented with AuNCs as laboratory standards for matrix-matched calibration by LA-ICP-MS

Fig. S6 shows the linear trend observed for the average concentration of Au determined in HRPEsv cells@AuNCs standards (expressed as fg of Au per cell) by conventional nebulization ICP-MS and the concentration of AuNCs used for supplementation of HRPEsv cells. **Fig. S7** collects the qualitative 2D-image ($^{197}Au^{+}$ intensity signals) obtained for the analysis of HRPEsv cells@AuNCs standards (25 µg mL^{-1} and 50 µg mL^{-1}AuNCs concentration) by LA-ICP-MS. Additionally, **Fig. S8** depicts the calibration graph used for determination of APOE and MT2A proteins in HRPEsv cells, representing the average $^{197}Au^{+}$ intensity per pixel for each cell (determined by LA-ICP-MS) *versus* the Au concentration of HRPEsv cell@AuNCs standards for each AuNCs supplementation condition (determined by ICP-MS).

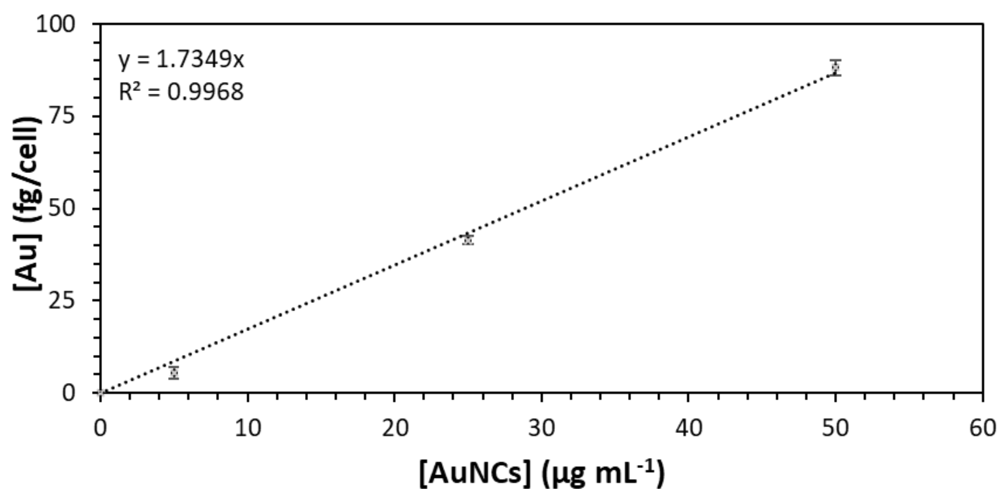

Figure S6. Relationship observed between the average concentration of Au (expressed as fg of Au per cell) determined in HRPEsv cells@AuNCs standards by conventional nebulization ICP-MS and the concentration of AuNCs (0, 5, 25 and 50 µg mL^{-1}) used for supplementation of HRPEsv cells. Error bars correspond to the standard deviation calculated for the analysis of around $1 \cdot 10^6$ cells per condition

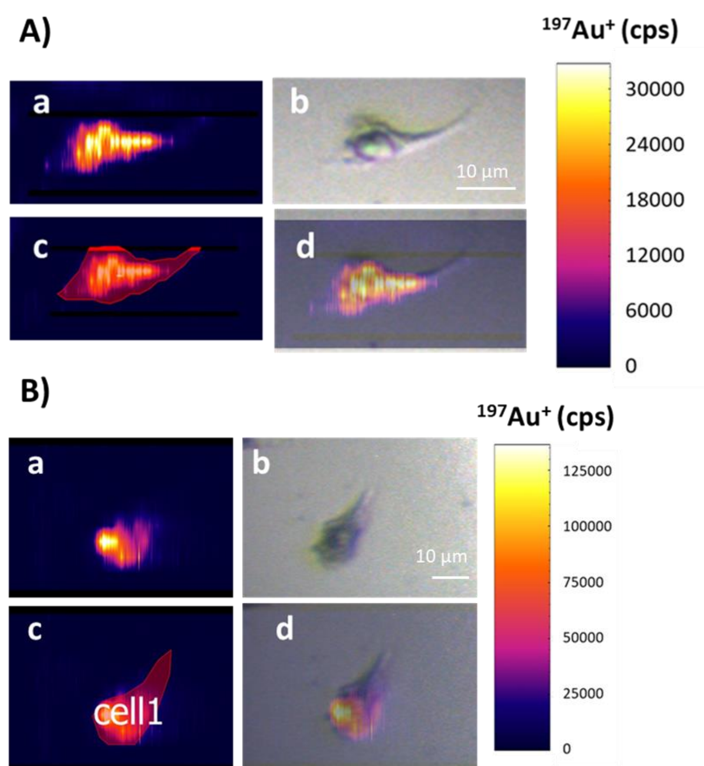

Figure S7. Analysis of HRPEsv cells@AuNCs standards by LA-ICP-MS. A) Supplementation with 25 µg mL^{-1} AuNCs, and B) Supplementation with 50 µg mL^{-1} AuNCs. In both cases, a) Qualitative 2D-image representing $^{197}Au^{+}$ intensity signals (cps), b) Optical image of the cells before LA sampling, c) Definition of the area corresponding to each individual marked in red, and d) Overlapping of $^{197}Au^{+}$ signal and optical image of the cells before LA sampling.

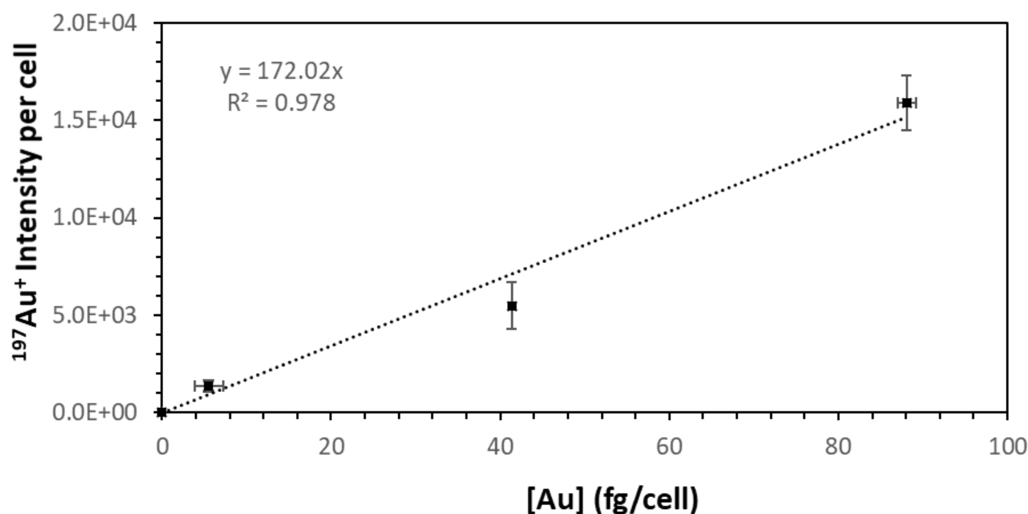

Figure S8. Calibration graph obtained for HRPEsv cells@AuNCs standards as matrix-matched calibration for the determination of proteins concentration in HRPEsv cells using LA-ICP-MS together with AuNCs-immunoprobe as specific tag. The average $^{197}Au^{+}$ intensity per pixel for each cell (determined by LA-ICP-MS analysis) was represented *versus* the Au concentration of HRPEsv cell@AuNCs standards for each AuNCs supplementation condition (determined by ICP-MS). Error bars - X axis correspond to the standard deviation calculated for the analysis of 30 individual cells per condition, whereas error bars - Y axis correspond to the standard deviation calculated for the analysis of around $1 \cdot 10^{6}$ cells per condition.

Determination of APOE and MT2A concentrations in HRPEsv cells by LA-ICP-MS using single-cell laboratory standards

Table S2 show the MT2A protein mass obtained by LA-ICP-MS for CT and IL1α–treated HRPEsv cells. Moreover, **Fig. S9** shows the qPCR results obtained of gen expression for APOE and MT2A proteins when HRPEsv cells were subjected to IL1α treatment for 48 h

Table S2. Experimental results obtained for the mass of MT2A per cell in CT and IL1α−treated HRPEsv cells analysed by LA-ICP-MS using AuNCs-immunoprobe as the elemental label and HRPEsv cells@AuNCs standards for calibration.

	Cell	Total Intensity (cps)	Cell size (pixels)	$^{197}Au^{+}$ Int./pixel	Au mass (fg/cell)	MT2A mass (fg/ cell)	MT2A mass (fg/cell)
MT2A CT	1	4.21E+06	1241	3.39E+03	19.70	1.3	2.1±0.9
	2	3.72E+06	835	4.45E+03	25.88	1.7	
	3	2.64E+06	600	4.39E+03	25.54	1.7	
	4	3.09E+06	635	4.87E+03	28.29	1.9	
	5	1.84E+06	334	5.50E+03	32.00	2.1	
	6	1.58E+06	418	3.79E+03	22.03	1.4	
	7	1.29E+06	516	2.49E+03	14.48	1.0	
	8	2.51E+06	567	4.42E+03	25.68	1.7	
	9	1.53E+06	538	2.85E+03	16.54	1.1	
	10	3.87E+06	943	4.11E+03	23.87	1.6	
	11	2.94E+06	994	2.96E+03	17.21	1.1	
	12	2.67E+06	847	3.16E+03	18.35	1.2	
	13	1.91E+06	537	3.57E+03	20.73	1.4	
	14	2.53E+06	598	4.23E+03	24.57	1.6	
	15	3.80E+06	935	4.06E+03	23.61	1.6	
	16	8.55E+06	1193	7.17E+03	41.66	2.7	
	17	1.15E+7	1350	8.55E+03	49.72	3.3	
	18	1.05e+07	1100	9.57E+03	55.65	3.7	
	19	1.08E+07	1230	8.82E+03	51.25	3.4	
	20	7.10E+06	841	8.44E+03	49.06	3.2	
	21	7.60E+06	980	7.76E+03	45.11	3.0	
	22	4.11E+06	435	9.46E+03	54.98	3.6	
	23	2.56E+06	398	6.44E+03	37.46	2.5	
MT2A IL1α	1	9.59E+06	620	1.55E+04	89.88	5.9	4.6 ±1.6
	2	8.01E+06	792	1.01E+04	58.81	3.9	
	3	3.85E+06	339	1.13E+04	65.97	4.3	
	4	9.58E+06	636	1.51E+04	87.56	5.8	
	5	2.63E+06	367	7.18E+03	41.72	2.7	
	6	6.34E+06	984	6.45E+03	37.51	2.5	
	7	1.14E+07	575	1.98E+04	115.33	7.6	
	8	4.40E+6	396	1.12E+04	65.36	4.3	
	9	1.38E+7	706	1.95E+04	113.58	7.5	
	10	3.19E+6	307	1.04E+04	60.40	4.0	
	11	7.85E+6	587	1.34E+04	77.69	5.1	
	12	5.66E+6	471	1.20E+04	69.87	4.6	
	13	7.77E+6	789	9.85E+03	57.25	3.8	
	14	5.16E+6	760	6.79E+03	39.46	2.6	

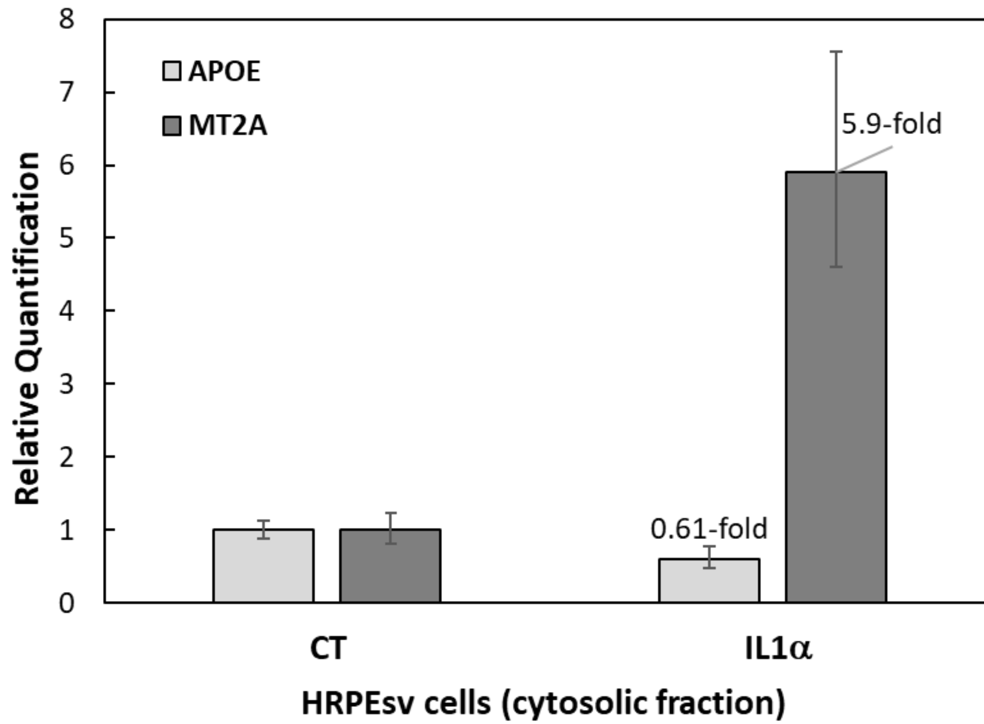

Figure S9. qPCR results of APOE and MT2A gene expression in HRPEsv cells under control and IL1α conditions, for 48 h. GUS was used as endogenous control. Error bars correspond to the standard deviation calculated for the analysis of three biological replicates.

REFERENCES

(1) P.J. Pao, E. Emri, S.B. Abdirahman, T. Soorma, H.H. Zeng, S.M. Hauck, R.B. Thomson, I. Lengyel, The effects of zinc supplementation on primary human retinal pigment epithelium, J. Trace Elem. Med. Biol. 49 (2018) 184-191. https://doi.org/10.1016/j.jtemb.2018.02.028

(2) A. Lores-Padín, P. Menero-Valdés, A. Rodríguez-Penedo, H. González-Iglesias, B. Fernández, R. Pereiro, Synthesis of size monodisperse water-soluble metal nanoclusters for protein quantification by elemental mass spectrometry, Mater. Proc. 4 (2021) 43. https://doi.org/10.3390/IOCN2020-07812

(3) M. Cruz-Alonso, L. Trapiella-Alfonso, J.M. Costa-Fernández, R. Pereiro, A. Sanz-Medel, Functionalized gold nanoclusters as fluorescent labels for immunoassays: Application to human serum immunoglobulin E determination, Biosens. Bioelectron. 77 (2016) 1055–1061. https://doi.org/10.1016/j.bios.2015.08.011

(4) A. Lores-Padín, M. Cruz-Alonso, H. González-Iglesias, B. Fernández, R. Pereiro, Bimodal determination of immunoglobulin E by fluorometry and ICP-MS by using platinum nanoclusters as a label in an immunoassay, Microchim. Acta 186 (2019) 705. https://doi.org/10.1007/s00604-019-3868-9

(5) M. Cruz-Alonso, B. Fernandez, M. García, H. González-Iglesias, R. Pereiro, Quantitative imaging of specific proteins in the human retina by laser ablation ICPMS using bioconjugated metal nanoclusters as labels, Anal. Chem. 90 (2018) 12145–12151. https://doi.org/10.1021/acs.analchem.8b03124

(6) T. Büchner, D. Drescher, H. Traub, P. Schrade, S. Bachmann, N. Jakubowski, J. Kneipp Relating surface-enhanced Raman scattering signals of cells to gold nanoparticle aggregation as determined by LA-ICP-MS micromapping. Anal. Bioanal. Chem. 406 (2014) 7003–7014. https://doi.org/10.1007/s00216-014-8069-0

(7) A. Lores-Padín, B. Fernández, L. Álvarez, H. González-Iglesias, I. Lengyel, R. Pereiro, Multiplex bioimaging of proteins-related to neurodegenerative diseases in eye sections by laser ablation - Inductively coupled plasma - Mass spectrometry using metal nanoclusters as labels, Talanta 221 (2021) 121489. https://doi.org/10.1016/j.talanta.2020.121489