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Evaluating pathological levels of intracellular cholesterol through Raman and surface-enhanced Raman spectroscopies

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Versatile methods for the quantification of intracellular cholesterol are essential for understanding cellular physiology and for diagnosing disorders linked to cholesterol metabolism. Here we used Raman spectroscopy (RS) and surface-enhanced Raman spectroscopy (SERS) to measure changes in cholesterol after incubating human fibroblasts with increasing concentrations of cholesterol-methyl- β -cyclodextrin. RS and SERS were sensitive and accurate enough to detect high levels of cholesterol in fibroblasts from patients affected by type C Niemann-Pick disease (NPC), a lysosomal storage disorder characterized by the primary accumulation of cholesterol. Moreover, SERS was able to distinguish between fibroblasts from different NPC patients, demonstrating higher accuracy than RS and standard fluorescent labeling of cholesterol with filipin III. We show that the type of gold nanoparticles used as signal enhancer surfaces in our SERS measurements are internalized by the cells and are eventually found in lysosomes, the main site of accumulation of cholesterol in NPC fibroblasts. The higher sensitivity of SERS can thus be attributed to the specific trafficking of our gold nanoparticles into these organelles. Our results indicate that RS and SERS can be used as sensitive and accurate methods for the evaluation of intracellular cholesterol content, allowing for the potential development of an optical detection tool for the ex-vivo screening and monitoring of those diseases characterized by abnormal modification in cholesterol levels.

Cholesterol is an essential lipid, which plays a crucial role in maintaining cell membrane integrity, signaling pathways, and various cellular processes¹. Levels of cellular cholesterol outside normal ranges are known to be implicated in several diseases, including cardiovascular diseases, metabolic disorders, neurodegenerative conditions and certain cancers^{2–6}. Accurate measurements of intracellular cholesterol can provide information about cellular health and function and allow for timely preventive or curative interventions, such as lifestyle adjustments and pharmacological therapies. Aside from more complex and labor and resource intensive methods based on gas chromatography or mass spectrometry⁷, one of the most common approaches to measure total unesterified (i.e. free cholesterol, the biologically active form of cholesterol not bound to proteins⁸) cellular cholesterol is based on fluorescent labeling with filipin III (a fluorescent detergent that specifically binds to free cholesterol⁹) and microscopy imaging. This technique allows researchers and clinicians to quantify free cholesterol levels within specific cell types and subcellular compartments^{10,11}. A recent alternative for staining cholesterol is the fluorescent recombinant perfringolysin O, a bacterial toxin that specifically binds cholesterol^{11,12}. Fluorescence based microscopy assays, however, can present some drawbacks and limitations, including intrinsic auto-fluorescence interference of biological samples, the need for cell fixation, signal sensitivity to environmental factors (e.g. pH, temperature, and solvent composition), and possible quenching and low-photostability of the fluorescence probe^{11,13}. In addition, filipin can also bind the ganglioside GM1¹⁴. Therefore,

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the need for alternative sensitive, more accurate and precise methods for cholesterol quantification would be beneficial for advancing diagnostic capabilities and evaluating potential therapeutic follow-up, facilitating timely and personalized interventions to enhance patient outcomes.

In this context, spontaneous Raman spectroscopy (RS) offers a potentially powerful alternative, being an optical, nondestructive and label-free solution for analyzing the chemical composition of materials, already widely used in materials science, biology, pharmacy and industry^{15,16}. This technique is based on the inelastic scattering of laser light: photons scattered by the sample may undergo frequency shifts that are associated with specific molecular vibrations. By examining the spectrum of scattered light, it is possible to recognize distinct Raman bands associated with known molecules and chemical bonds, allowing the investigation of sample composition. This method has already been widely used in biology for studying lipids¹⁷, proteins and nucleic acids¹⁸ in cells, body tissues and fluids. In particular, several studies applied RS for investigating cholesterol distribution in tissues, primarily in atherosclerotic plaques^{19–22}, but also in neoplastic tissues such as colorectal, lung and brain cancers^{23–25}. Other papers targeted or detected cholesterol within lipid membranes²⁶, clusters of extracellular vesicles²⁷, and in individual cells of plants²⁸, animals²⁹ and humans³⁰. For example, Schultz (2011) observed a cholesterol gradient in rod cells by looking at the intensity of its 695 cm⁻¹ band, while H. Autefage et al. (2015) measured changes in the cholesterol content of stem cells using Raman spectrum clustering analysis.

However, despite its potential, Raman spectroscopy also has limitations, the most crucial being that the Raman effect is very weak, with a lower probability of occurring than, for example, fluorescence absorption. This is particularly relevant in biological materials³¹, where the presence of many intrinsic fluorophores can cause significant fluorescence background interference and hinder the detection of Raman signals. Moreover, since biological systems are often a complex mixture of biochemicals, recorded spectra are superpositions of all their vibrational modes, and the assignment of Raman bands to specific molecules may become a challenging task³².

The problems associated with RS can be overcome with the introduction of surface enhanced Raman scattering (SERS), an optical process that enhances the Raman signal by several orders of magnitude (typically 10⁶–10⁷) by taking advantage of localized surface plasmons (LSPs) of metallic nanostructures and their interactions with the electromagnetic field scattered by the sample³³. Thanks to the easiness and affordability of their synthetic procedures as well as their established biocompatibility, gold nanoparticles can be exploited as enhancing surfaces³⁴. The adoption of SERS further increases the sensitivity and specificity of RS by utilizing nanostructured surfaces that amplify its signals. This enhancement is particularly advantageous when dealing with low concentrations of analytes within cellular environments. Therefore, researchers could leverage these techniques to gain insights into cholesterol dynamics within cells, facilitating real-time monitoring and advancements in our understanding of cellular processes^{35,36}.

In this study, we compared RS and SERS capabilities to detect changes in cholesterol concentration: firstly, in wild-type fibroblasts (WT FB) incubated with increasing concentrations of water soluble cholesterol (cholesterol-methyl- β -cyclodextrin, β -CD-chol), and then in fibroblasts derived from patients affected by Niemann-Pick disease type C (NPC, OMIM #257220). NPC is a rare neurodegenerative metabolic disorder characterized by the primary accumulation of free cholesterol in lysosomes and late endosomes, as a consequence of mutations in the NPC1 and NPC2 proteins, which are thought to be involved in the cellular transport of cholesterol³⁷. In addition to the presence of the most typical clinical symptoms (such as splenomegaly and ataxia), the diagnosis of NPC at the biochemical level is mainly established using fibroblasts cultures from skin biopsies and subsequent imaging analysis of cholesterol content by fluorescent filipin III staining, and potentially followed by genetic analysis. For neurological cases without organ alterations, diagnosis is often delayed and might not even be made. In addition, in 15–20% of the cases, the result of the filipin test may be of problematic interpretation¹⁰. Alternative biomarkers have recently been proposed for non-invasive, blood-based diagnostics of NPC³⁸. In our study, the cholesterol levels of NPC cells were measured with RS and SERS and qualitatively quantified through ratiometric scoring. To our knowledge, this is the first time that these two methodologies have been successfully applied for the study and diagnosis of this pathology. The results obtained by the two techniques were compared with those retrieved from fluorescence imaging and filipin III staining. Moreover, an additional advantage of our approach is the exquisite targeting of the gold nanoparticles used for SERS to the lysosomal compartment, a remarkable advantage considering that the lysosome is the main site of accumulation of cholesterol in NPC. Our findings underscore how RS and SERS could represent relatively fast and label-free methods for measuring the cholesterol content of cells, particularly in cases displaying lysosomal accumulation.

Methods

Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O); trisodium citrate dihydrate (C₆H₅O₇Na₃·2H₂O); Poly(ethylene glycol) 2-mercaptoethyl acetic acid (SH-PEG-COOH, Mn 7500); N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC); N-Hydroxysuccinimide (NHS); Rhodamine 110 (Rh110), Filipin III; all organic solvents were purchased from Merck (Darmstadt, Germany) and used without further purification. DMEM medium, Fetal Bovine Serum (FBS), penicillin/streptomycin solution (P/S), Opti-MEM, LysoTracker™ Red DND-99, Hoechst 33,342, Leibovitz's L-15 were purchased from ThermoFisher Scientific (Waltham, MA, USA); cholesterol-methyl- β -cyclodextrin was purchased from Merck. Niemann-Pick cells (NPC) of two patients (NPCp1 and NPCp2, carrying the p.Thr1205Asnfs*53 fs/p.Thr1205Asnfs*53fs³⁹ and p.Q991Rfs*15/p.Q991Rfs*15⁴⁰ mutations, respectively) were purchased from the "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases" (G. Gaslini Institute) – Telethon Genetic Biobank Network⁴¹. No patients were directly involved.

Gold nanoparticles synthesis and functionalization

Gold nanoparticles (AuNPs) were synthesized by the seeded-growth process described by Yuan et al.⁴². The solution of 15 nm sphere-shaped AuNPs was prepared by adding 1.5 mL of 30 mM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (1%) to 48.5 mL of boiling and stirring MilliQ (stirring 400 rpm, 250 °C). After 10 s, 4.5 mL of 38.8 mM sodium citrate solution were added to the solution. Solution was stirred under heating for 15 min, and then stirred without heating for 30 min obtaining citrate-capped gold nanospheres dispersed as colloidal phase in aqueous solution.

To obtain fluorescence-labeled AuNPs, a functionalization procedure with Rhodamine110 (Rh110) was implemented. First, bifunctional SH-PEG-COOH molecules were exploited in a ligand-exchange reaction to replace the citrate layer onto gold surfaces and subsequently for further conjugation with Rh110. The molar ratio between SH-PEG-COOH and AuNPs was kept constant at 5000, as optimized in our previous work^{43,44}.

To this end, the concentration of AuNPs was determined according to a formula reported by Liu et al.⁴⁵. Briefly, AuNPs solution was diluted to 1 nM and mixed with an equal volume of a 0.01 M aqueous solution of PEG and stirred at room temperature overnight. The so-obtained pegylated-nanoparticles (PEG-AuNPs) were centrifuged at 25 °C—10,000 rpm and redispersed in milliQ water at 1 nM. Carboxylic pendant moieties of PEG-AuNPs were activated by adding 0.4 mM EDC and 0.1 mM NHS (final concentration) for 30 min at room temperature, followed by a subsequent addition of different amount of aqueous solution of Rh110 to obtain various final concentrations ranging from 10 μM to 100 μM . The reaction was stirred overnight, then fluorescent-nanoparticles (fluo-NPs) were centrifuged at 25 °C—10,000 rpm and redispersed in the appropriate buffer for the incubation with cell culture^{43–45}.

Cell cultures and imaging

To evaluate the enrichment of cholesterol in cells, WT FB were plated in DMEM medium (plus 10% FBS and 1% P/S) in 12-well plates containing glass coverslips at a 30,000 cells/well density and, 24 h after plating, were incubated for 3 h at 37 °C with increasing concentrations of water soluble cholesterol (cholesterol-methyl- β -cyclodextrin, β -CD-chol) ranging from 0.2 to 1.0 mg/mL. Then, the cells were fixed with 4% PFA, rinsed with PBS (plus 0.5 mM MgCl_2 and 0.8 mM CaCl_2), and permeabilized with 0.075% Triton X. After rinsing with PBS and blocking with 4% BSA-PBS, the cells were incubated with 0.25 mg/mL of Filipin III for 24 h at 4 °C to stain the cholesterol. After rinsing with PBS and water, the cells were imaged with a custom-made wide-field epifluorescence microscope equipped with an oil-immersion objective (Nikon Plan Apo TIRF 100x/1.45), a DC4100 4-Wavelengths LED Source (Thorlabs, Newton, NJ, USA) and a heating chamber. A 405 UV-LED with a Blue Fluorescent Protein excitation and emission filter set (Chroma, Taoyuan, Taiwan) was used⁴⁶.

The same procedure described above was used to evaluate the cholesterol content in FB from NPC patients and from WT control as already shown previously⁴⁶. Importantly, primary skin fibroblasts observed here were not exposed to the LDL-enriched medium typically used in the standard ‘filipin test’ used for biochemical diagnosis of NPC.

To demonstrate the colocalization between AuNPs and lysosomes, WT FB were plated as reported above and incubated for 30 min at 37 °C in DMEM medium (plus 3% FBS and 1% P/S) containing 5 nM AuNPs labeled with Rhodamine 110 (AuNPs-Rh), 50 nM LysoTracker™ Red DND-99 (for lysosomes staining), and 10 $\mu\text{g}/\text{mL}$ Hoechst 33,342 (for nuclei staining). After washing with PBS, the incubation medium was replaced with the Leibovitz's L-15, a medium designed for supporting cell growth in the absence of CO_2 equilibration. The analysis of AuNPs, lysosomes and nuclei-derived fluorescence intensities was performed after excitation at 488, 561 and 405 nm, respectively, using a Nikon Eclipse TE300 C2 laser scanning confocal microscope (CLSM) (Nikon, Tokyo, Japan) and a Plan Fluor 100 $\times$ 1.49 NA oil immersion objective. Optical sections at median planes of the cells (1024 $\times$ 1024 pixels) were taken for each sample and analyzed using the Fiji software. Images were acquired using sub-saturation settings.

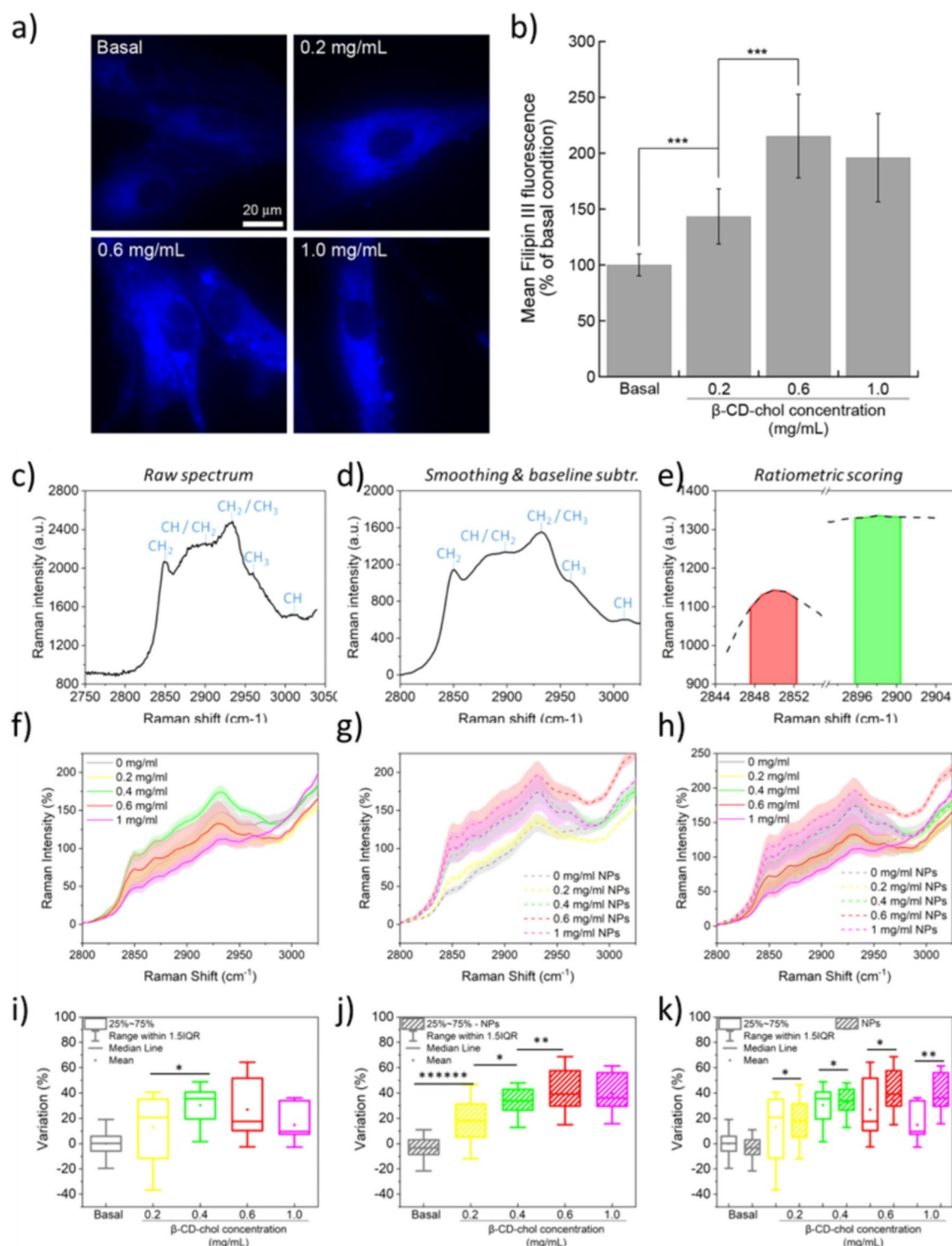
A similar colocalization analysis between AuNPs and lysosomes was performed for FB from NPC patients and from WT control.

In order to properly quantify the degree of colocalization between AuNPs and lysosomes, the JACoP plugin⁴⁷ available under the Fiji software was used to determine the Pearson's correlation coefficient⁴⁸ (an estimate of the covariance in the signal levels of two images, analyzing them pixel-by-pixel) and the Manders' overlap coefficients⁴⁹ (an indicator of pixel fraction of the image 1 that overlaps with the image 2, and vice versa).

Characterization techniques

The plasmonic properties of gold NPs colloidal solution before and after functionalization processes were acquired in the range from 400 nm to 900 nm with a UV-Vis-NIR spectrophotometer (Lambda 950 instrument, Perkin Elmer, Waltham, MA, USA). UV WinLab (Perkin Elmer, Waltham, MA, USA) and Origin software were used to acquire spectra and to process data, respectively. The hydrodynamic dimensions, the polydispersity, and the zeta potential were characterized by Dynamic Light Scattering (DLS) analysis performed with a Malvern Zetasizer Nano series ZS90 (Malvern, Worcestershire, UK). Measurements were performed with a fixed scattering angle of 90°, at 25 °C. Each sample was measured three times and each measurement consisted of about 30 acquisitions. Cumulating statistics were used to measure the hydrodynamic diameter and polydispersity. For ζ -potential, each sample was measured three times and each measurement consisted of 100 acquisitions. Data were then processed with Origin software. The NPs morphology in terms of size and shape was characterized by transmission electron microscopy (TEM, CM 12 PHILIPS, Amsterdam, Netherlands)^{43,44}.

Raman spectra were collected with a conventional inverted micro-Raman setup (XploRA PLUS Confocal Raman Microscope, Horiba, Kyoto, Japan), consisting of a 532 nm laser (Coherent, Santa Clara, CA, USA) and a spectrometer with a focal length of 500 mm, equipped with a 1800 lines/mm grating and Plan-Apochromat 60 $\times$ water-immersion objective (Nikon, Tokyo, Japan; NA = 1.27, WD = 0.17 mm). The incident laser power on the sample was about 20 mW. The scattered light was detected by a cooled CCD camera. Raman-SERS spectra were



recorded in the wavenumber range of 2750–3040 cm^{-1} , the acquisition time was 20 s and the measurements were repeated six times for spectral averaging; 2–3 spectra were collected from as many positions outside each cell nucleus, and averaged together. The spectral range was selected after preliminary measurements on water-soluble cholesterol revealed significant Raman emission between 2800 and 3000 cm^{-1} , particularly a prominent peak at $2845 \pm 10 \text{ cm}^{-1}$ (Supplementary Figure S1); on the other hand, the “fingerprint region” (600–1800 cm^{-1}) was avoided due to significant background signal from the glass coverslip beneath cells. Cells were analysed in PBS 1x in 12-well plates containing glass coverslips (0.01 mm of thickness). For SERS measurements, 1 nM of AuNPs (6×10^{11} particles/ml) were previously incubated overnight at 37 $^{\circ}\text{C}$, then rinsed thrice with PBS 1x enriched with Ca^{2+} and Mg^{2+} to remove the unbound NPs^{43,44}. This specific concentration was chosen as an optimal concentration that maximises SERS enhancement while minimising potential cytotoxic effects.

◀ **Fig. 1.** Enrichment of β -CD-chol in WT FB. Cells were analyzed in basal conditions and after 3 h of incubation with 0.2, 0.6, and 1.0 mg/mL of β -CD-chol. Cells were then probed with the fluorescent dye Filipin III and imaged with wide-field epifluorescence microscopy (a,b). Filipin III fluorescence intensity significantly increases with the increase of β -CD-chol concentration up to 0.6 mg/mL, reaching a fluorescence signal plateau between 0.6 and 1.0 mg/mL. > 30 cells were analyzed for each condition (b). Error bar S.D. Student's t-test *** $p \leq 0.001$. Pre-processing and analysis of Raman spectra. Each raw spectrum (c) was restricted to the range between 2800 cm^{-1} and 3026 cm^{-1} and smoothed via Savitzky-Golay filtering, with its lowest intensity value subtracted from the whole spectrum (d). The highlighted Raman bands were assigned according to the data reported in¹⁷ (see also Supplementary Table T1). Finally, the overall intensities of two Raman bands centered at 2850 cm^{-1} and 2898 cm^{-1} were computed (e), with their ratio being used as a score for estimating lipid concentration in cells. Raman-SERS analysis of WT FB cells enriched with β -CD-chol in the range $0 - 1\text{ mg/mL}$. Mean RS (f), SERS (g) and both RS and SERS (h) spectra $\pm$ standard errors (SEs) of enriched cells; ratiometric scores measured from RS (i), SERS (j) and both RS and SERS (k) spectra of enriched cells, expressed as percentage variation from the mean control (i.e. 0 mg/mL added, without NPs) value.

Data analysis

The collected Raman spectra were pre-processed and analyzed through ratiometric scoring. The spectral range was centered on CH bands between 2800 cm^{-1} and 3026 cm^{-1} , a Savitzky-Golay filtering (1st order polynomial, with 2 side points) was implemented for noise reduction, and then the lowest intensity value was shifted to 0 in order to account for possible spurious contributions to the baseline of the recorded signal. Ratiometric scoring consisted in calculating the ratio between photon counts collected at two different spectral bands of each spectrum, and specifically: the intensity @ $2849.9 \pm 2.5\text{ cm}^{-1}$ (corresponding to CH_2 vibrations in lipids¹⁷) divided by the intensity @ $2898.1 \pm 2.5\text{ cm}^{-1}$ (CH/CH_2 bonds in proteins and lipids).

Such a method is a well-known analytical tool for Raman spectra. For example, Veluthandath et al. (2024)⁵⁰ correlated the intensity ratio between CO and CC bands to sphingomyelin's concentration in liposomes; Rensonnet et al. (2023)⁵¹ exploited CH , CH_2 and CH_3 bands to discriminate six fatty acids within cellular lipid droplets; Jamieson et al. (2018)⁵² were able to correlate Raman ratiometric scores recorded on food oil samples to their fatty acids properties (degree of saturation and chain length). In this particular case, we chose ratiometric scoring for the following reasons. By using two bands, the score is independent of absolute photon counts, hence of all the parameters (e.g. laser power and collection efficiency) that can differ from one experimental setup to another. Moreover, since these bands can be associated with lipids and proteins, their ratio allows different concentrations of lipids to be highlighted – both with and without NPs – in NPC and WT control cells. In conclusion, they provided the best discriminatory power and a reasonable interpretation of the results.

Finally, Two-Sample t-tests were performed among all cell classes in order to determine if their scores were statistically different ($p\text{-value} \leq 0.05$).

For a more general presentation of our results, ratiometric scores and spectral intensity in the following figures are reported not as absolute values (e.g. photon counts), but as percentage variations from, respectively, average score and average intensity of non-enriched WT FB cells.

Results and discussion

Proof-of-concept: RS and SERS measures of fibroblasts enriched in cholesterol

As a preliminary experiment to assess whether RS and SERS are capable of revealing and discriminating different levels of cholesterol, we used them to monitor the uptake of increasing concentrations of β -CD-chol (in the range of $0.2 - 1.0\text{ mg/mL}$) by wild-type fibroblasts (WT FB). The results were compared to those obtained with filipin III labeling of cholesterol in fixed and permeabilized cells. The analysis of images acquired with an epifluorescence microscope revealed a significant increase in the total cholesterol levels proportional to the increase of the β -CD-chol concentration up to 0.6 mg/mL , while no further increase was observed at 1.0 mg/mL (Fig. 1a and b). In comparison, vibrational spectra were acquired from the same unlabeled cells incubated with and without citrate-capped AuNPs for RS and SERS analysis, respectively (Fig. 1f-k). We specifically examined the high wavenumber region between 2800 and 3026 cm^{-1} , since this part of the spectrum includes several Raman bands associated with different types of lipids⁵³. The recorded spectra were characterized by an overlap of multiple peaks, and the intensity of both the Raman and Raman-SERS signal increased together with the concentration of cholesterol. In order to accurately quantify the differences between spectra recorded at different concentrations, each collected Raman spectrum was pre-processed and analyzed through ratiometric scoring (Fig. 1c-e, see the Data analysis section for further details). In brief, the intensity ratio between two main contributions at 2850 and 2900 cm^{-1} (grossly corresponding to lipid and protein contents, respectively⁵⁴⁻⁵⁷ was calculated for each class of cholesterol level, together with the corresponding percentage change (Fig. 1i-k) from the control (no β -CD-chol added). Data reported in Fig. 1i show that RS is unable to distinguish between the different enrichment levels with statistical significance, except for the comparison between 0.2 and 0.4 mg/mL ($p < 0.05$). By contrast, SERS gives better results in terms of discrimination, as reported in Fig. 1j: all the variations between 0 and 0.6 mg/mL are statistically different. The $p\text{-value}$ between 0.6 and 1 mg/mL is > 0.05 instead, which is consistent with the filipin III fluorescence data showing saturation for these values.

RS and SERS evaluation of altered cholesterol content in fibroblasts from NPC patients

Once we had demonstrated the ability of RS and SERS to discriminate between different levels of cholesterol, a second experiment was carried out applying similar RS and SERS analysis on FB isolated from NPC patients (NPCp1 and NPCp2). Initially, we measured cholesterol accumulation in NPC and WT cells using the filipin

III labeling protocol: as expected, the analysis of wide-field microscope images revealed a significant increase (~3-fold) in the cholesterol levels in both NPCp1 and NPCp2 samples compared to the WT (Fig. 2a, b). Then, RS and SERS spectra were acquired following the same conditions optimized for the cholesterol enrichment experiment. As shown in Fig. 2c, slight alterations of the RS intensity between the normal (WT) and pathological conditions (NPCp1 and NPCp2) can be observed in correspondence to the cholesterol bands. Such a finding suggests the effectiveness of RS and ratiometric scoring to significantly discriminate between the presence of normal and abnormal levels of cholesterol, with an average ~20% increase observed in patients' scores (WT-NPCp1: $p < 0.0001$; WT-NPCp2: $p < 0.00001$; Fig. 2e). However, the RS mean spectra of the two patients (red line and blue line in Fig. 2c) almost overlap, precluding any differentiation between them ($p > 0.05$; Fig. 2e).

On the other hand, as demonstrated above, the internalization of AuNPs within cells substantially improved the ability to differentiate between different cellular cholesterol concentrations through optical spectroscopy. Similarly to what has been observed with RS, SERS mean spectra presented different features and intensities for each analyzed sample (see black, red and blue lines in Fig. 2d). We observed a wider separation between NPCp1 and NPCp2 ratiometric scores from the WT counterparts, with the former being on average 30% and 45% higher than the latter, respectively. Moreover, statistical differences were found not only between control and patients' scores (WT-NPCp1: $p < 10^{-7}$; WT-NPCp2: $p < 10^{-9}$; Fig. 2f), but also between the two patients ($p < 0.001$), indicating a slight increase in the cholesterol content (Fig. 2f) in NPCp2 with respect to NPCp1.

AuNPs uptake and localization in lysosomes

The better performance of SERS in discriminating minor cholesterol variations could be explained by the intracellular trafficking of gold NPs. Owing to their size of a few nanometers, this type of NPs can easily enter the cells through micropinocytosis, an endocytosis process in which the NPs are grasped into small vesicles which eventually evolve in or fuse with lysosomes⁵⁸. Considering that the main cellular hallmark of NPC is the massive accumulation of cholesterol and other lipids in late endosomes and lysosomes³⁷, the internalized AuNPs could potentially localize in the proximity of accumulated cholesterol, at the level of the lysosomes. The specific enhancement of the cholesterol Raman peaks could then be explained by the interaction and close proximity of the cholesterol molecules to the surface of the NPs, which facilitates a stronger signal specifically at the sites of cholesterol accumulation in the lysosomes. To demonstrate this hypothesis, AuNPs were first labeled with the green fluorescent probe Rhodamine 110 (Fig. 3a) and then their distribution was followed after incubation with WT and NPCp1 cells. Rhodamine conjugation was first assessed through extinction measurements. After synthesis, citrate-gold nanospheres (Cit-NPs) were characterized by an absorbance spectrum showing a characteristic resonance peak centered at around 520 nm. As a consequence of their surface modification, the extinction coefficient experienced a minor red-shift of at least 4 nm, due to the change of chemical environment at NPs surface after the conjugation (Fig. 3b). As expected, the size of NPs was 14 ± 3 nm (in agreement with the images obtained with TEM in Fig. 3c) and resulted in a slight increase to 50 ± 11 nm after molecules grafting, as graphically represented by the autocorrelation curves and histograms reported in Fig. 3d–e. Moreover, the retaining of the characteristic fluorescence peak centered at 530 nm was exploited to characterize the successful attachment of the dye molecules to the surface of the NPs (Fig. 3f).

Fluorescent Rh110-AuNPs at the same concentration used for all the experiments reported above (1 nM) were incubated overnight with the cells. Image analysis with confocal microscopy reveals a very high degree of colocalization between lysosomes and Rh110-AuNPs both in WT and NPCp1, while no overlapping signal was observed between lysosomes and the Rh probe alone (i.e. in the absence of AuNPs), indicating that the localization of AuNPs-Rh into the lysosomes is fully attributable to the AuNPs (Fig. 4).

Pearson's coefficient values, calculated to quantify the degree of colocalization of AuNPs and lysosomes, indicated a high degree of colocalization (0.672 ± 0.082 and 0.684 ± 0.156 for WT FB and NPCp1, respectively). The Manders' coefficients M1 (fraction of Rh110-AuNPs overlapping with lysosomes) and M2 (fraction of lysosomes overlapping with Rh110-AuNPs) corroborated further this result ($M1 = 0.672 \pm 0.082$ for WT FB and 0.734 ± 0.193 for NPCp1, and $M2 = 0.653 \pm 0.125$ for WT FB and 0.755 ± 0.100 for NPC1, respectively).

Conclusions

The results presented herein demonstrate that RS and SERS, two label-free techniques, are capable of monitoring and quantifying the amount of cholesterol in human fibroblasts either enriched with exogenous cholesterol or deriving from NPC patients, in which the accumulation of cholesterol is a pathological hallmark. Notably, the use of AuNPs and SERS provides a higher precision in assessing cholesterol accumulation than either RS or fluorescence imaging with filipin III labeling, allowing the distinction between different levels in WT FB as well as between two fibroblast populations deriving from different NPC patients. In the NPC experiment, the better performance of SERS is possibly a consequence of the specific accumulation of the AuNPs in lysosomal compartments, in which the excess cholesterol is mainly stored. Aside from contributing to research into NPC, our approach could be of interest for the monitoring and therapeutic follow-up of other rare disorders that present alterations in the cholesterol levels, such as lysosomal acid lipase deficiency (LALD)⁵⁹, MEGDEL syndrome⁶⁰, Smith-Lemli-Opitz syndrome (SLOS)⁶ and Tangier disease⁶¹. These diseases are associated with defects in the biosynthesis (SLOS being the most common one), with the lysosomal accumulation of cholesterol esters (LALD), with the intracellular trafficking (MEGDEL syndrome) or with the export of cholesterol outside the cells (Tangier)⁶. Notably, similar increases in filipin III staining levels were found in fibroblasts deriving from NPC, SLOS and Tangier⁶. RS and SERS could represent alternative label free approaches, not susceptible to fluorophore bleaching and other artefacts caused by intrinsic fluorescence, for studying these diseases.

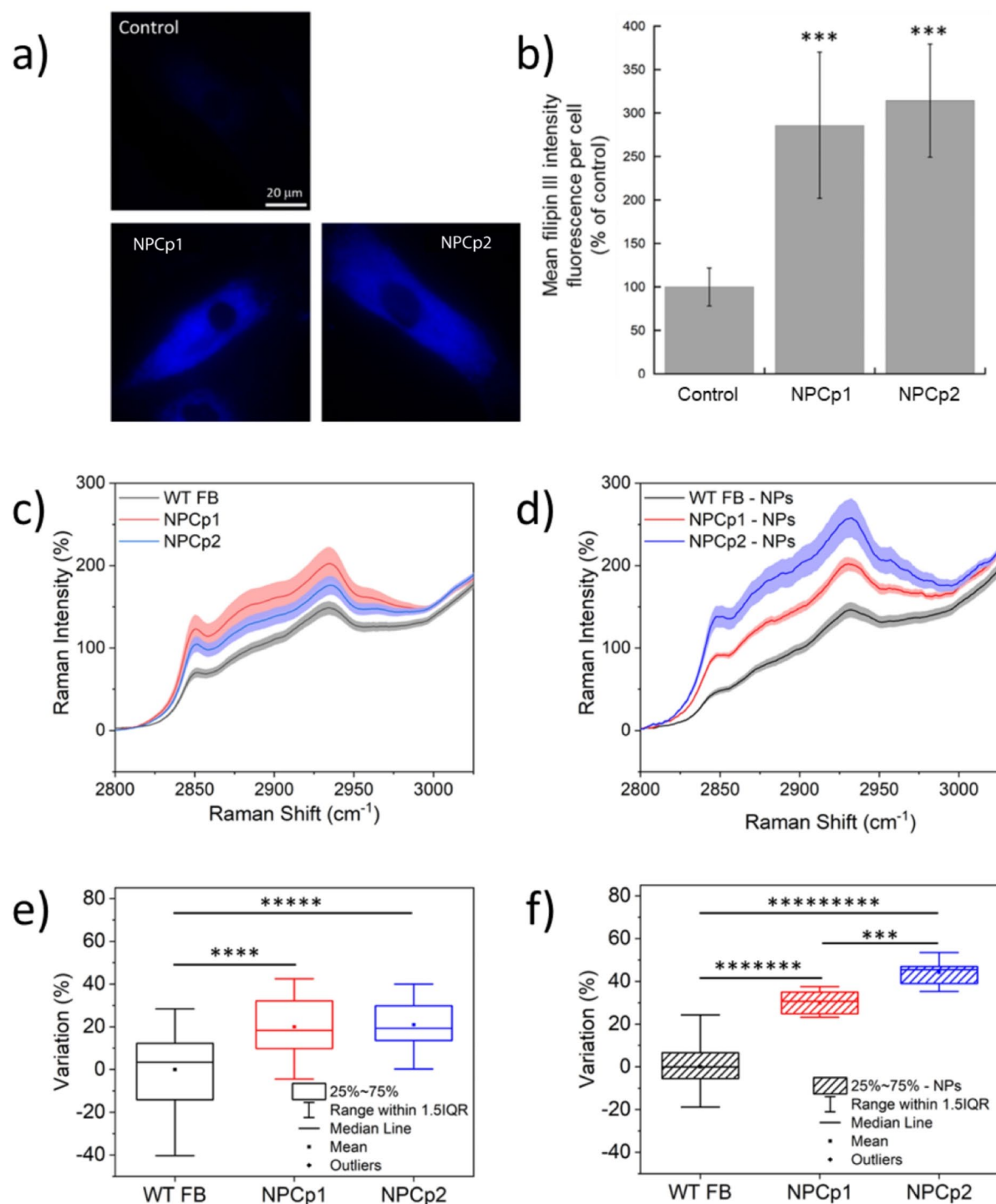


Fig. 2. Analysis of cholesterol content in FB from NPC patients. Thawed primary cultures of FB isolated from WT control and NPC (NPCp1 and NPCp2) patients were cultured, fixed, permeabilized, labelled with Filipin III and (a) imaged with wide field epifluorescence microscopy (a). Significantly higher values of Filipin III were found analyzing NPCp1 and NPCp2 images compared to control, indicating a rise in cellular cholesterol content (b). > 30 cells were analyzed for each sample. Error bar S.D. Student's t-test *** $p \leq 0.001$. Raman-SERS analysis of FB cells from NPC patients (c–f). Mean RS (c) and SERS (d) spectra $\pm$ SEs of WT and NPC' cells; ratiometric scores measured from RS (e) and SERS (f) spectra of WT and NPC' cells, expressed as percentage variation from the respective mean control values.

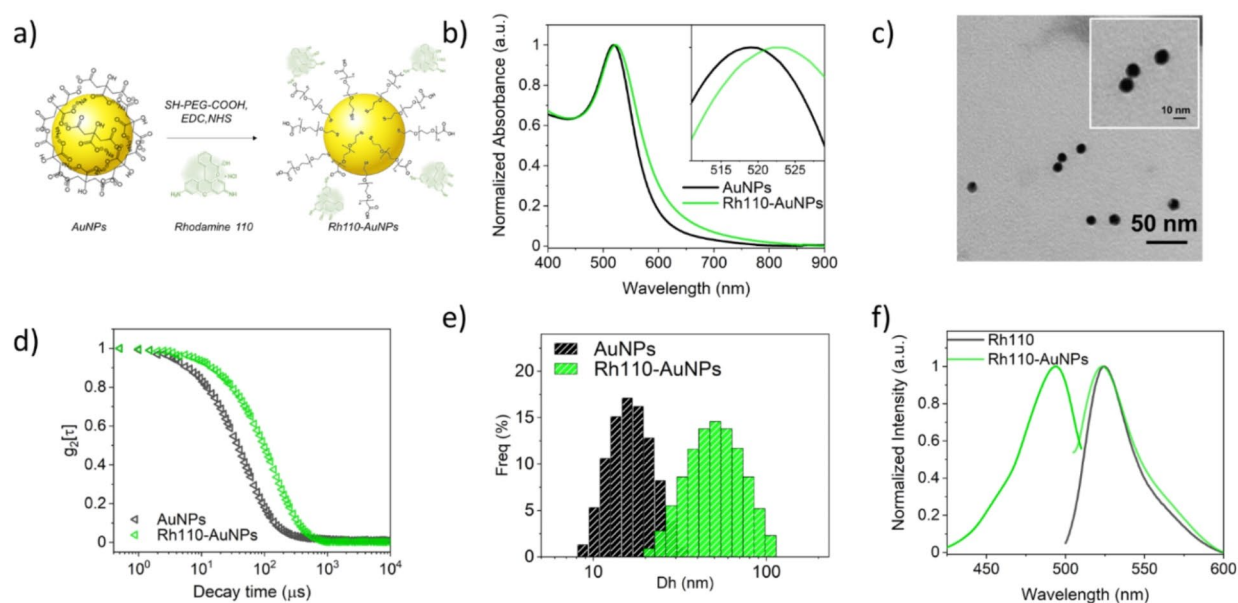


Fig. 3. Fluorescent Rh110-AuNPs characterization. Graphical scheme depicting Rhodamine 110 conjugation to AuNPs (a). UV-Visible absorption spectra of AuNPs (black line) and Rh110 conjugated AuNPs (green line). Inset shows a magnification in the range 510–530 nm (b). TEM images of AuNPs (c). Autocorrelation curves in function of the decay time (d) and the calculated hydrodynamic diameters of AuNPs (black dot/histogram) and Rh110-AuNPs (green dot/histogram) (e). Fluorescence emission spectrum of Rhodamine 110 solution (black line) and absorbance and fluorescence spectra of Rh110-AuNPs (green line) (f).

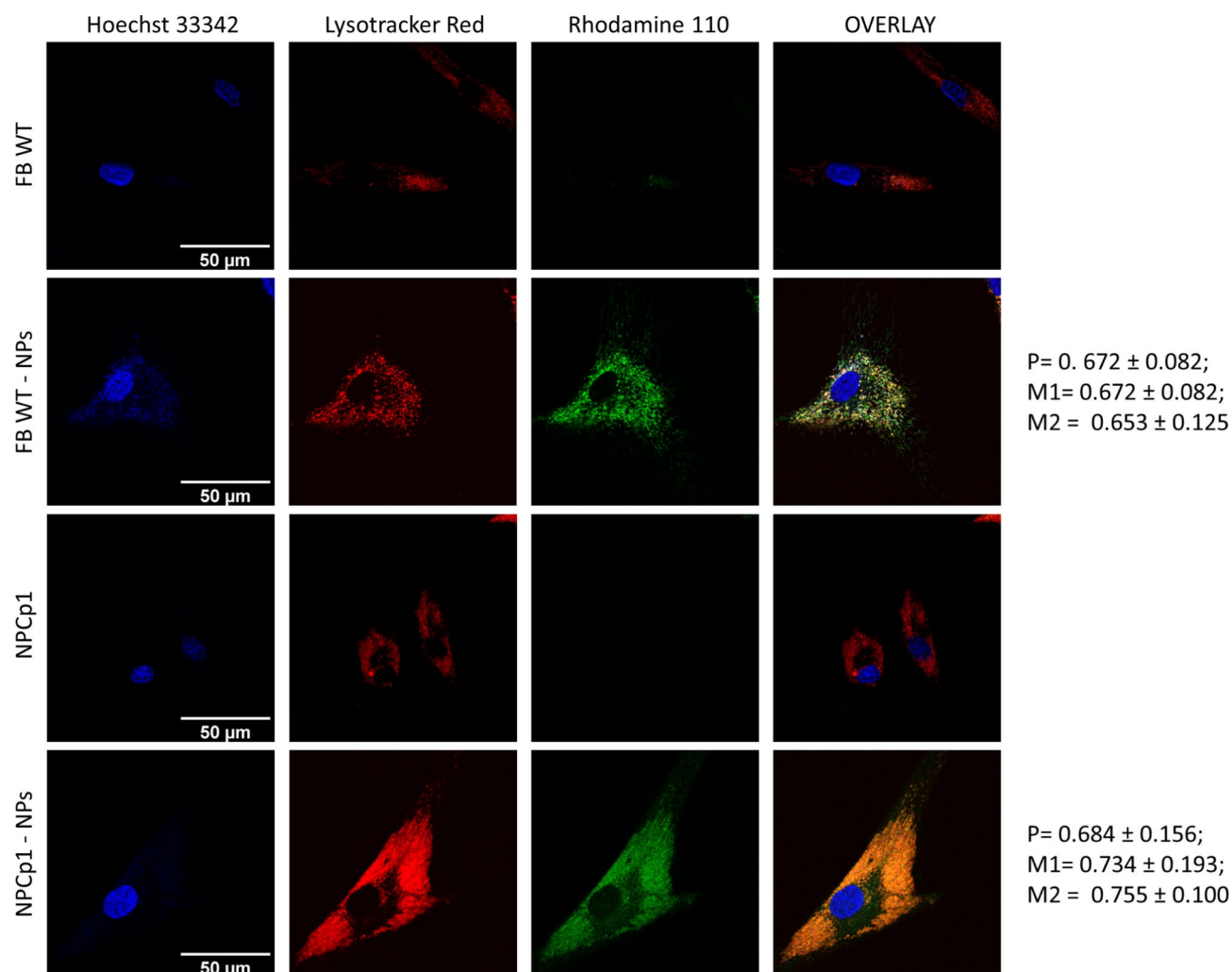


Fig. 4. Specific targeting of AuNPs in lysosomes. WT and NPCp1 fibroblasts were incubated overnight with 1 nM Rh110-AuNPs or Rh110 probe, and imaged by confocal microscopy after co-labeling with LysoTracker™ Red DND-99 and Hoechst. Scale bar 50 μm. The Pearson's coefficient (P) values and the Manders' coefficients M1 (fraction of Rh110-AuNPs overlapping with lysosomes) and M2 (fraction of lysosomes overlapping with Rh110-AuNPs) are also reported.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

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Author contributions

M.C., E.B., C.D., R.C., Ca.C., Cl.C. contributed to the conception or design of the work; E.B., C.D., R.C., Cl.C., F.M. contributed to the acquisition, analysis, or interpretation of data; M.C., E.B., C.D., R.C., Ca.C., Cl.C., A.M., F.P. contributed to have drafted the work or substantively revised it; M.C. supervised the work.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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