Quantifying lipids in human cells using Raman spectroscopy

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Abstract
Omics methods should deliver information on cellular composition as well as their content and localization in cells. This is particularly important for lipids, both saturated and unsaturated fatty acids, which regulate key metabolic, signaling, and toxic processes. Furthermore, differences in local concentration can determine cell fate: activating endoplasmic reticulum stress, exacerbating lipotoxicity, or promoting metabolic adaptation. By using deuterated fatty acids as Raman probes (Rp), we can determine their local concentrations within cells, taking into account, for example, accumulation in lipid droplets, ER or membranes. Monitoring the incubation time and distribution of Rp allows for the assessment of the rate of lipid uptake and accumulation in the cell. Therefore, RS spectroscopy methods can be used to study the kinetics of lipid transport and metabolism, key in fluxomics. We quantitatively calibrate the C–D band intensities using deuterated lipid standards and compare the RS results with LC-MS and MF data to verify the quantitative accuracy of the Raman method. We demonstrate that despite using µM concentrations of dPA and dOA acids in the medium, local concentrations inside cells (especially in lipid droplets) can reach mM levels which demonstrates the importance of a local, not just total, lipid detection method.

Introduction
The importance of fatty acid localization and distribution in cells (lipid metabolism, signaling); local concentrations are a key metabolic and functional parameter, and high local concentrations are crucial for biological function and effects. Application in lipotoxicity studies (high local concentrations of saturated fatty acids lead to activation of ER stress, PERK/eIF2α, autophagy, and even apoptosis), by localizing them, one can predict cell susceptibility to death induced by high local lipid concentrations. [1] Tracking cancer metabolism, where cells strongly alter lipid metabolism by synthesizing fatty acids de novo or utilizing exogenous ones, can also be explored. [2] Cell preference for saturated or unsaturated fatty acid accumulation can be considered a marker of the cancer phenotype. [3], [4] The location and concentration of fatty acids can help in the selection of drugs that inhibit the elongation of fatty acid carbon chains, their synthesis or the activation of oxidation, which allows for testing the effectiveness of treatment by monitoring the distribution of fatty acids in time and space.[5]

Materials and Methods
Sample preparation
HeLa (Human cervical cancer cell, Sigma Aldrich) were cultured in MEME medium (Minimum Essential Medium Eagle, Sigma-Aldrich,), supplemented with 5 % heat-inactivated fetal bovine serum (FBS, Lonza), 1% Penicillin-Streptomycin (Sigma-Aldrich), 1% L-Glutamine solution (Sigma-Aldrich,) and 1% MEM Non-essential Amino Acid Solution (Sigma-Aldrich, cat. No M7145). Cell cultures were maintained at 37 °C in a 98 % air / 5 % CO_2 atmosphere, passaged every 4 to 5 days when 80 % confluency was reached. HeLa cells were seeded on sterilized CaF_2 slides with a diameter of 12 mm and 1 mm thickness (Crystran, UK) at a density of 50,000 cells per well in a 24-well plate and left for 24 hours to adhere. On the day of treatment, the culture medium was replaced with a fresh medium. The d_{31}-Palmitic Acid (d_{31}-PA, Sigma Aldrich) solution was added directly to the medium at 200 µM and 50 µM concentrations for 6 h or 24 h incubation time. To facilitate fatty acid transport

through the cell membrane, d_{31}-PA was first saponified using NaOH (Sigma Aldrich) at 70 °C and then conjugated with bovine serum albumin (BSA, fatty acids-free, Sigma Aldrich). After incubation, cells were gently rinsed with Dulbecco`s Phosphate Buffer Solution (DPBS, Sigma Aldrich) and fixed for 10 minutes at room temperature with 2.5% glutaraldehyde (Sigma Aldrich). The fixed cells were kept in DPBS at 4°C until measurements were taken. Just before measurements, the cells were rinsed with room-temperature DPBS.

Raman measurements
Raman imaging of HeLa cells was performed using a confocal Raman microscope, WITec Alpha 300 (WITec GmbH, Ulm, Germany), equipped with air-cooled solid-state lasers operating at 488 nm, 532 nm, and 633 nm, along with a CCD detector (Andor Technology Ltd, Belfast, Northern Ireland). The excitation wavelength of 488 nm was utilized to excite the cell samples, with a laser power of approximately 50 mW (measured before the objective). Data processing was conducted using WITec Project Plus software.

Fluorescence measurements
HeLa cells were incubated for 24 h with fluorescent palmitic acid (NBD, Sigma-Aldrich) at concentrations ranging from 5 µM to 100 µM then fixed with 2.5% glutaraldehyde and stained with Hoechst (Sigma-Aldrich) and CellMask Red (Sigma-Aldrich). Fluorescence measurements were subsequently performed using the Axio Observer.D1 (Carl Zeiss) fluorescence microscope.

Results

1. Quantification of the Raman Probe

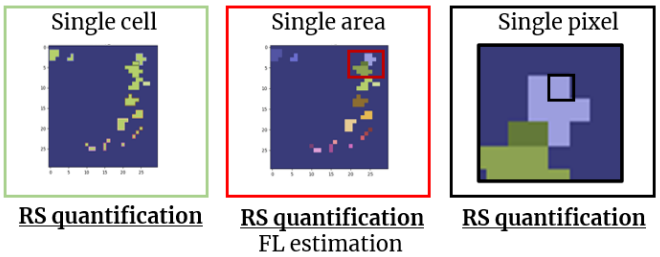

Fig. 1 Three levels of quantification of the Raman probe in cells.

The scheme above (Fig. 1) illustrates the concept of multi-scale determination of the concentration of a deuterated Raman probe by combining data from fluorescence microscopy and Raman spectroscopy. To determine Rpr concentration, we established three analytical strategies: single cell Rp concentration focused on the average dPA concentration in a single cell; single area analysis combining fluorescence imaging and Raman measurements presents analysis averaged spectra from chemically similar regions and use fluorescence palmitic acid as a reference and single-pixel analysis based on analysis that allows quantification of dPA concentration in chemically homogeneous regions at the single pixel level through chemical mapping. All strategies require a dPA calibration curve and a precise method of segmenting the obtained images.

A single cell
The global intracellular concentration of the Rp per cell is inherently challenging to interpret. Deuterated fatty acids are internalized through multiple uptake routes, including passive diffusion and channel- or carrier-mediated transport. Once inside the cell, they enter numerous metabolic pathways: they are incorporated into membrane lipids, stored as esterified species in lipid droplets, trafficked through endosomal compartments, transported into the nucleus, or directed toward mitochondrial β-oxidation for energy production.
Consequently, Raman spectroscopy (RS) reports a composite signal arising from all intracellular pools of deuterium-labeled lipids and their metabolic derivatives. This integrated signal includes contributions from both the exogenous deuterated fatty acids supplied during incubation and from

downstream metabolites retaining the deuterium label. As a result, the apparent intracellular concentrations derived from RS substantially exceed the concentrations present in the culture medium (50–200 µM), reaching 60–500 mM when expressed as a global per-cell concentration.

Selected cellular area
The concentration of deuterium within spectrally distinct intracellular regions, here referred to as the concentration of individual subcellular areas, reveals that the distribution of the deuterium label is highly heterogeneous. Deuterated species can be detected in regions enriched in proteins as well as in areas dominated by esterified lipids. Such sublocal concentration estimates can be derived from Raman spectroscopic data and interpreted in a semi-quantitative manner. Although these values cannot be directly converted to absolute concentrations, they can be compared with the spatial distribution of a fluorescent palmitic acid analogue used for cell incubation.
However, this comparison is not ideal, as dPA and FL-PA represent chemically distinct probes. Moreover, the fluorophore attached to palmitic acid significantly increases cytotoxicity than dPA and photobleaching over time.

A single pixel
Local quantification is feasible exclusively through Raman spectroscopy (RS). It is performed by summing the integrated C–D band intensities within subcellular regions that exhibit similar Raman spectral profiles. Determining the local concentration of the Rp using this approach directly addresses the central question posed in this study.

2. Data analysis

Calibration model based on RS
The first step was to prepare a calibration curve for RS. Deuterated fatty acid was dissolved in various concentrations of oleic acid and, after being applied to a CaF2 slide, spectra were measured for selected concentrations ranging from 0 mM to 3477 mM for dPA. The first step was to prepare a calibration curve for RS. Deuterated fatty acid was dissolved in various concentrations of oleic acid and, after being applied to a CaF2 slide, spectra were measured for selected concentrations ranging from 0 mM to 3477 mM for dPA.

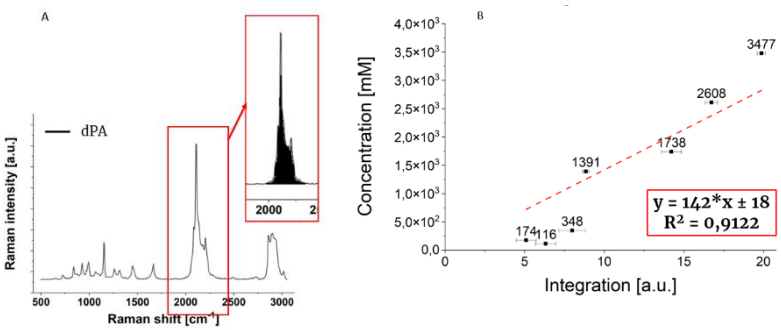

Fig. 2 The integration diagram for the deuterium band and the calibration curve for dPA acid. Trend lines were fitted to the obtained results, and equations describing the relationship between concentration and integration of the deuterium band were derived. Measurements were repeated three times, and for each point, measurements were also made at several locations.

Quantification analysis
<u>Single cell quantification</u>

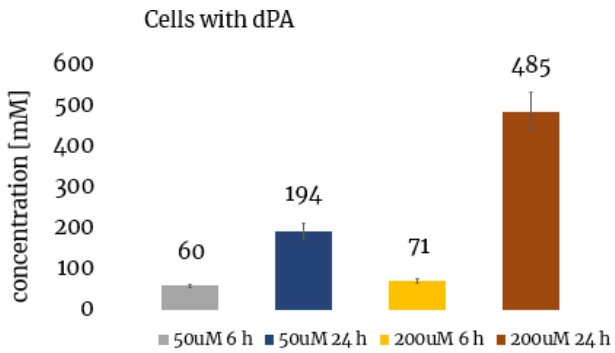

Fig.3 Single cell concentration for HeLa cell incubated with 50 uM and 200 uM dPA at 6 h and 24 h.

The mean concentration after 6 hours of incubation with 50 µM and 200 µM dPA was nearly the same. With longer incubation times, the mean concentration of dPA increased significantly, and at the higher incubation concentration it was approximately 2.5 times greater than at the lower concentration. Extending the incubation time leads to a significant increase in dPA concentration, and this effect is much stronger at the higher applied dose, indicating a clear time- and dose-dependent relationship.

<u>A selected cellular area</u>

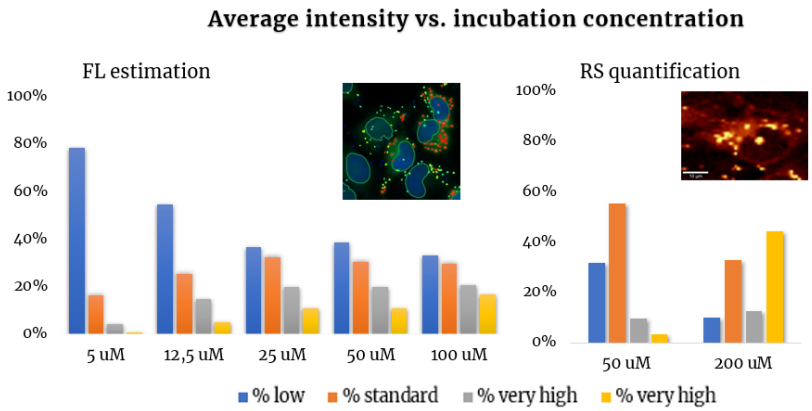

Fig.4 The percentage distribution of regions with comparable intensities and concentrations for Fluorescence Microscopy and Raman spectroscopy.

The most notable finding was the comparison of the percentage share of deuterium-rich regions between FL and RS. In FL, we observe a decrease in low-intensity deuterium-rich areas accompanied by a slight increase in higher-intensity regions. This may reflect greater accumulation of the dye, potentially within lipid droplets, as incubation concentration increases. In RS, lower concentrations are dominated by regions with mid-range intensity values, whereas higher concentrations lead to a clear shift toward strongly deuterium-rich areas.

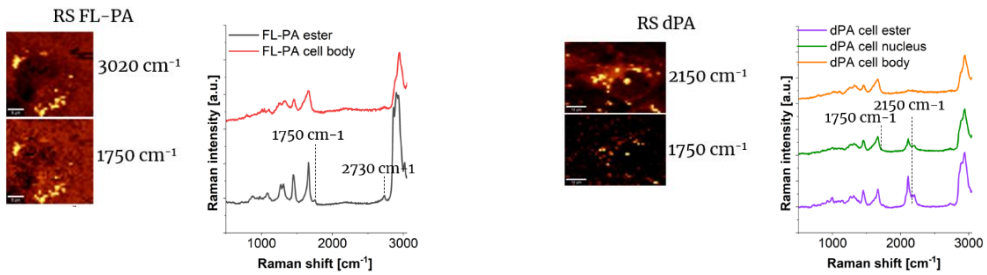

Fig. 5 Raman imaging and spectrum for FL-PA and dPA in HeLa cells.

Differences in the percentage distribution of regions with comparable intensities and concentrations may arise from several factors. First, FLPA and dPA are two distinct Rp. FLPA is considerably more

toxic to cells and therefore cannot be used at high concentrations. RS measurements indicate that FLPA accumulates mainly in regions rich in esters, most likely in lipid droplets. Although FLPA provides higher sensitivity, its usefulness is limited by reagent toxicity. In contrast, RS analysis of dPA shows dPA-rich regions not only in lipid droplets but also in other cellular compartments, such as the nucleus. This suggests that our measured dPA levels reflect its incorporation throughout the entire cell, rather than being restricted solely to lipid droplets.

Single-pixel

This approach shows how dPA concentration values are distributed at the level of individual pixels. The key observation is that the concentration range derived from the calibration curve reaches a maximum of ~390 mM for the lower incubation concentration, whereas for the higher concentration, under the same incubation time, it reaches nearly 1600 mM. The mean pixel-level concentration across the entire cell population also depends on the incubation dose and increases accordingly. These results highlight the strong heterogeneity of the measured samples and emphasize that, in RS-based measurements, spectra are collected from diverse cellular regions and organelles, which naturally contributes to the wide spread of concentration value s.

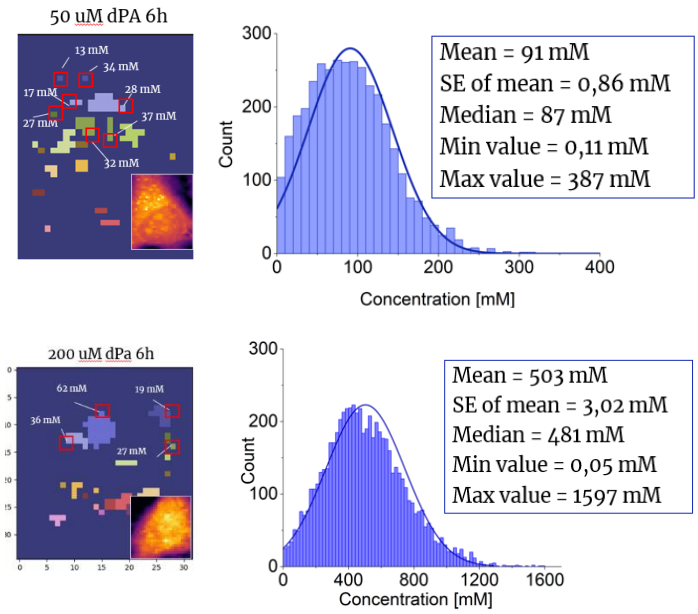

Fig. 6 Single pixel distribution of concentration of dPA after 6 h of incubation with 50 µM and 200 µM dPA.

Conclusion

Raman spectroscopy using deuterated fatty acid enables quantitative, subcellular determination of metabolite concentrations, providing insight not only into their presence but also into their functional roles in metabolic dynamics and cellular signaling. Notably, micromolar incubation concentrations can generate millimolar levels of Rp within cells, thereby offering strong signal contrast for intracellular mapping. Because Raman spectroscopy is non-invasive, it supports time-resolved kinetic studies without compromising cell integrity, allowing researchers to monitor local concentration fluctuations in real time in response to external stimuli such as pharmacological agents. This capability opens new ways for fluxomics and dynamic lipid research, facilitating the identification of critical nodes in lipid flux by tracking molecular kinetics and spatial redistribution within living cells. Nevertheless, several limitations remain. Raman spectroscopy is inherently semi-quantitative and therefore requires appropriate reference methods for accurate calibration. Further validation across a wider range of cell models is essential, as is methodological refinement to enhance sensitivity, quantitation, and compatibility with various metabolite classes. Together, these developments will be crucial for advancing Raman-based metabolic imaging toward more comprehensive and fully quantitative analyses of cellular metabolism.

Acknowledgements
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