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Towards Single Organelle Lipidomics in Live Cells

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Abstract

Detailed studies of lipids in biological systems including their role in cellular structure, metabolism and disease development, comprise an increasingly prominent discipline called lipidomics. However, the conventional lipidomics tools, such as mass spectrometry, cannot investigate lipidomes until they are extracted, and thus cannot be used neither for probing the lipids distribution, nor for studying in live cells. Furthermore, conventional techniques rely on the lipid extraction from relatively large samples, which averages the data across the cellular populations and masks essential cell-to-cell variations. Further advancement of the discipline of lipidomics critically depends on the capability of high-resolution lipid profiling in live cells and, potentially, in single organelles. Here we report micro-Raman assay designed for single organelle lipidomics. We demonstrate how Raman microscopy can be used to measure the local intracellular biochemical composition and lipidome hallmarks – lipids concentration and unsaturation level, cis/trans isomers ratio, as well sphingolipids and cholesterol levels in live cells, with a submicron resolution, which is sufficient for profiling of subcellular structures. These lipidome data were generated by a newly developed Biomolecular Component Analysis software, which provides a

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Author Contributions

A.N.K., A.P. and A.L. conceived, designed and performed the experiments presented here; A.N.K., A.B., A.P., A.R., M.G., A.L. discussed the results. P.N.P. and M.L. provided conceptual input and supervised the research. A.N.K. and A.P. wrote the manuscript with contributions from all other coauthors.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Raman spectra of the same sample (Bovine serum albumin water solution) excited by 633 nm and 785 nm lasers; Raw Raman spectra of the same nucleolus (the same cell) measured using 100x and 63x objective lenses and corresponding parameters of confocality; Raman spectrum of HeLa AG cellular lipids in the range between 1100 and 1800 cm^{-1} ; Structures of oleic and linoleic acids, N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine, chicken egg sphingomyelin and corresponding Raman spectra in the range between 1370 and 1800 cm^{-1} ; Raman spectra of typical lipid droplet in HeLa cell, N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine, and Cholesteryl linoleate; Raman spectra of AG lipids in HeLa cells, extracted by BCAbox; Representative raw and pre-processed Raman spectra of cellular organelles and corresponding residual and lipid Raman spectra; One-way ANOVA for sets of organellar lipidome parameters.

The authors declare the following competing financial interest(s): P.N.P. is the owner of ACIS, LLC, a company developing BCAbox. A.N.K. and A.P. are employees of ACIS, LLC.

shared platform for data analysis among different research groups. We outline a robust, reliable and user-friendly protocol for quantitative analysis of lipid profiles in subcellular structures. This method expands the capabilities of Raman-based lipidomics towards the analysis of single organelles within either live or fixed cells, thus allowing an unprecedented measure of organellar lipid heterogeneity, and opening new quantitative ways to study the phenotypic variability in normal and diseased cells.

Graphical Abstract

INTRODUCTION

Lipidomics, although recently emerged, has already branched out as a discipline, investigating a family of lipid molecules in biological systems. The lipids content is very diverse and includes up to staggering hundred thousands of chemically distinct entities¹. Lipid molecules are small and, while often exhibiting only minor variations in the chemical structure, are pre-disposed to hinder the development of chemically selective probes for labeling of specific lipid species in their locations. Hence, research in this field relies on the mass-spectrometry technique, which provides unsurpassed chemical detection specificity. Extensive data have elucidated the role of lipids in biological structure, metabolism, and cell signaling, and reveal these biomolecules as biomarkers of various diseases^{2–6}.

However, mass-spectrometry cannot map lipid species in tissues and relies on lipid extraction. These lipids are typically extracted from relatively large samples containing up to several million cells, because high concentrations of analytes are required. Thus, despite the availability of these methods, the cell-to-cell variations in the distribution of lipids are poorly characterized. While the heterogeneity of cells in normal tissues and tumors has been thoroughly investigated by genomics and proteomics, only a few lipidomics approaches have advanced beyond the conceptual stage. Google Scholar illustrates this challenge by returning over six thousand references for “single cell genomics” while searching for “single cell lipidomics” yielded just 57 research articles at the time this manuscript was being written. Studies of lipid variations in single cellular organelles or evolution of lipid profiles in cells and subcellular structures over time, are even more complicated. Data acquired in this field would be intriguing for analysis, and may advance the understanding of molecular regulation

of a cell, thereby supporting the development of new technologies capable of molecular recognition at high 3D resolution and in live samples.

The inherent shortcomings of mass-spectrometry techniques can be overcome by optical vibrational, including Raman, spectroscopic methods. The Raman optical process relies on the inelastic scattering of incident light on molecular vibrations with either a loss or gain of the energy, equal to that of a particular vibrational mode. Thus, if the probing photons are monochromatic, the scattered photons compose a spectrum with either longer, Stokes shift, or shorter, anti-Stokes shift, wavelengths, bearing information about vibrational modes, which, in turn, relate to chemical bonds of matter. It has to be emphasized that the intensity of Raman scattering linearly depends on the analyte concentration. Thus a routine concentration calibration of Raman spectrometer permits measurements of absolute biomolecular concentrations in the samples. Such spectroscopic analytical techniques have breakthrough potential in biomedical research, primarily because of the capability of quantitative biomolecular probing *in situ* and in real time, with submicron spatial resolution^{7–17}.

Recent advances in confocal spontaneous Raman spectrometry, Coherent Anti-Stokes Raman Spectroscopy and Stimulated Raman Spectroscopy are providing opportunities for *in situ* compositional analysis and comprehensive lipids characterization^{18–25}, allowing for the first time local probing of concentration and unsaturation level of lipids in live cells^{26–30}. However, due to the complex nature of cellular Raman spectra, they have been mostly limited to the analysis of lipid aggregates (*e.g.*, cytoplasmic lipid droplets), where concentrations of lipids are high, and the presence of other biomolecules does not produce any significant spectral distortions. At the same time, in the complex biomolecular mixtures typically found in the cellular organelles, any quantitative analysis of lipids is troublesome because of the overlap of their characteristic peaks in the Raman spectra.

Here, we introduce a platform for characterization of lipids in single cellular organelles *in situ*. In the core of our approach is the Biomolecular Component Analysis (BCA) of Raman spectra (Figure 1). Briefly, the protocol involves localization of organelles by fluorescence probes (Step 1 and 2), acquisition of Raman spectra in the labeled organelles (Step 3), spectral deconvolution and analysis of cellular components with the help of our newly designed BCABox software (Step 4). Described here procedure selectively defines the lipids component within the acquired organellar spectrum. Once the lipid spectrum is established, the lipid concentration, lipid unsaturation parameters, the trans/cis isomers ratio, and the cholesterol and sphingolipids levels can be obtained (Step 5).

Cancer biology constitutes an important application of Raman spectroscopy. For example, the unsaturation parameter of lipids is of utmost importance in estimating the stemness potential of a tumor-initiating cell³¹. Given the rarity of these cells in the tumor microenvironment, the application of BCA to identify them is valuable, since very few techniques are capable of cellular classification without any additional tagging procedure, and without any external perturbation of the cellular biological machinery in a single cell or at the organelle level. The proposed algorithm is incorporated into a BCABox, currently a

beta version of the Raman spectrometry BCA software, which will make our approach universally accessible.

EXPERIMENTAL SECTION

BCA platform description.

The Raman optical process is scattering of probing photons on molecular vibrations with a loss or gain of the energy equal to that of a particular vibrational mode. Thus, if the excitation light is monochromatic, the scattered photons compose a spectrum with either longer (Stokes shift) or shorter (anti-Stokes shift) wavelengths, bearing information about vibrational modes, which, in turn, relate to chemical bonds of matter. The linear nature of this optical process provides a simple algorithm for the acquisition of quantitative information from spectral data, *i.e.* a concentration calibration procedure for Raman spectrometer supplies absolute measurements of biomolecular concentrations. To collect organellar Raman spectra *in situ*, we have explored a confocal Raman microscope, which provided a submicron spatial resolution of probing intracellular area.

To analyze collected spectra, we have used the BCABox, a stand-alone software package for biomolecular component analysis. In contrast to Principal Component Analysis, the most popular implicit method in Raman multivariate bioanalysis⁷, BCA, as one of the explicit methods, is based on an accurate spectral fit of the measured Raman spectrum to a model spectrum, generated by a summation of the weighted spectra of the basic molecular components¹⁵. The spectral weights are considered as the specific contributions of the basic spectra into the resulting spectrum, and relate directly to the concentrations of basic macromolecules. The residual profile between the model and the real spectrum is usually used as a merit of the model quality, which can be estimated by comparing the residual intensities over the significant wavelength range, with a standard error produced during the measurement of the high-concentrated solution of protein. Detailed information on BCABox software application *in situ* can be found in our recent study on HeLa cultured cells³².

In our experience, the most variative biomolecular components in the cellular composition are lipid species. In this consideration, analysis of the residual spectrum by monitoring of intensities in wavelength ranges assigned to lipid fingerprints, is one of the possible ways to detect the presence specific lipid components, like cholines or cholesterols. As the result of this analysis, subtraction of weighted profiles of proteins, DNA, RNA, and glycogen from the background free organellar spectrum, provides organellar lipid spectrum for further extraction of lipidome parameters.

The Raman microscope.

We have used the DXR2 Raman microscopy system (Thermo Fisher Scientific, Madison, WI), which includes a red laser source unit with the power of excitation sufficient to obtain the required BCA quality for Raman spectra in biological samples (see "Excitation source" section). To visualize cellular organelles, this system is equipped with a fluorescence Illuminator (5-UR7005) with a green fluorescence cube (488/561EX) and a mercury lamp (X-Cite 120 PC, Photonic Solutions, Inc., MA). The set-up computing system was equipped

with a OMNIC software for dispersive Raman (Thermo Fisher Scientific, Madison, WI) and BCAbox (ACIS, LLC, Buffalo, NY).

Excitation source.

A 633 nm@70mW single frequency laser diode (ROUSB-633-PLR-70-1, Ondax, CA) provided ~30mW of excitation at the sample. The laser was chosen to achieve an optimal balance between the photodamage of live cells, triggered by a tightly focused laser beam, and the ratio between the signal and the background generated during the Raman spectrum measurement. It has to be considered that shorter wavelengths of excitation, *e.g.*, 488 nm and 532 nm, used for Raman microscopy, are known to be phototoxic to live cells³³. Longer wavelengths, *e.g.*, 785 nm and 1064 nm, are not phototoxic and do not produce detrimental autofluorescence background from biosamples, but generate larger instrumental background (most likely from glass elements, see Figure S1), which is detrimental for the accuracy of BCA.

Microscope objective.

The choice of a microscope objective is crucial in Raman microspectrometry of cellular organelles. For a confident BCA result, the size of the probing laser beam waist should be comparable to that of the measured organelle. We used a Plan N oil immersion 100^x ($N_a=1.25$) Olympus objective lens, which provides a submicron XY spatial resolution and produces a lower background signal compared to that of a water immersion lens with a comparable numerical aperture (see Figure S2).

Device Calibration.

A wavelength calibration option, which is responsible for yielding accurate Raman shift for each point of the spectrum, is built in the OMNIC, commercial software of DXR2, and this procedure is initiated periodically when necessary.

Concentration calibration of spectrometer.

To provide BCAbox analysis in absolute biomolecular concentrations, we have calibrated the spectrometer by measuring the Bovine Serum Albumin solution in water (Sigma A3912), and then used the ratio of 1003 cm^{-1} peak intensities as a correction coefficient for known concentration. For this procedure, we prepare an aqueous solution of Bovine serum albumin (BSA) of known concentration (Sigma-Aldrich, St. Louis). We used 255 mg/mL of BSA as an optimal concentration for better accuracy of the calibration. To ensure that solution does not contain solid particles of BSA, we filtered the solution by a 1 μm pore filter. To verify the concentration of the filtered BSA solution, we evaporated water from a known volume of solution and weighted the solid BSA fraction. For calibration measurement, we used a solution sealed between the microscope slide and the coverslip using a secure seal imaging spacer. Measured intensity difference ΔI between the maximum at $\sim 1003 \text{ cm}^{-1}$ and the local minimum at 1020 cm^{-1} was used for calculation of coefficient for absolute concentrations correction to that shown in BCAbox section: $k = \frac{\Delta I}{60.2}$.

Measurement of Raman spectrum.

For Raman setup with an upright configuration and oil immersion objective lens, we covered the well with the cells at the bottom of the dish by 22 mm circle cover glasses (VWR Scientific, Chicago, IL) and sealing with a waterproof silicon sealant. This procedure allows the application of the oil immersion objective lens, without disturbing the cells (i.e. upside down position of dish on the microscope stage). We also found that sealed cells, if there are no air bubbles inside the cell volume, demonstrated excellent viability, suitable for measurements over a long sessions (>8 hours). Conveniently, post measured cells could be kept for further incubation, after removing the sealant and circle coverslip, and replacing the medium.

Before the first measurement, the dish should be kept untouched for at least ~5 min. This time is necessary for temperature and mechanical stabilization to avoid any mechanical Z-on-axis movement of the bottom glass during measurements.

For spectral acquisition from specific organelles, the ER, AG, and mitochondria were labeled by green fluorescence organelles trackers, as described above. For visualization of the green fluorescence signal, the fluorescence cube 488/561EX was used. For the acquisition of each spectrum, the excitation laser was focused on an individual organelle. To ensure the absence of vibration, thermal drift, or other motion in our system during experiments, we visually verified the XYZ position of the cell before and after each measurement. While this experimental arrangement allows pinpointing diverse subcellular structures, the resolution of confocal Raman microscopy does not always ensure precise probing of pure organellar structures. This limitation is due to (a) a potential difference between the confocal probing volume and the dimensions of organelles, and (b) a non-uniform structure of organelles. In particular, Raman probing of the ER yields information on both, the ER lumen and the surrounding cytosol. The contribution of cytosol is presented in Raman spectra of the mitochondrion as well. Nevertheless, the differences of the Raman spectra in the probing volumes of distinct organelles are significant, and are, most likely, associated with the biomolecular microenvironment of the specific organelle.

To meet the signal/noise ratio requirement and to generate good quality BCA while avoiding unwanted phototoxicity, the accumulation parameter 6×20 seconds was established for each spectrum acquisition.

Typical raw and pre-processed Raman spectra of stained Apparatus Golgi, Endoplasmic Reticulum and mitochondrion of live HeLa cells acquired by DXR2 Raman microscope are shown in Figure S3.

Spectrum analysis by BCAbox.

BCAbox works with a single raw (un-preprocessed) Raman spectrum measured in the cell on a glass-bottom dish. The program consists of three main blocks: the pre-processing unit (correction of wavelength shift, background subtraction, and baseline correction), the nonlinear least squares routine unit, and the input/output data unit. Input data includes the following parameters: (i) measured cellular spectrum; (ii) choice of cellular organelle where this spectrum was measured (nucleus, nucleolus, mitochondrion, endoplasmic reticulum,

apparatus Golgi for growing cell; chromosome or cytoplasmic areas for mitotic cell); and (iii) choice of live or fixed (either formaldehyde or ethanol) cell. The toolbox delivers the following outputs: (i) Background free Raman spectrum, (ii) Residual spectrum for estimation of modeling quality, (iii) Weight coefficients for five biomolecular components (proteins, DNA, RNA, lipids, glycogen). (iv) The spectrum of organellar phospholipids, (iv) Basic parameters for lipidome in organelles, including lipid unsaturation, trans/cis ratio of stereoisomers, sphingolipids, and cholesterol content. The saved results of the spectrum analysis were located in the “Output” folder of the *BCAbox*|*build*|*Application*| directory in the .txt format.

Code availability.

Proprietary BCAbox software is available with the purchasing of DXR2 (Thermo Fisher Scientific, Madison, WI) and Confotec™ MR350 (SOL instruments Ltd, Minsk, Belarus) Raman microscopes. The method for organellar lipids measurements is under patent pending protection (U.S. Pat. Appln. No. 62/696,656).

Cell culturing and sample preparation.

HeLa and U251 cells (ATCC) were grown in luminescence free 35 mm glass bottom dishes (Fisher Scientific Co, Hanover Park IL), and cultured in Advanced DMEM (Life Technologies Corporation, Grand Island NY), supplemented with 3% fetal calf serum, glutamax antibiotic-antimycotic solution (Life Technologies Corporation, Grand Island NY) at 37 °C in a humidified atmosphere containing 5% CO₂. R132C mutation of isocitrate dehydrogenase was introduced with the help of lentivirus under puromycin selection. To ensure accurate BCA, the cells were transferred into an optically transparent medium, red-free DMEM (Life Technologies Corporation, Grand Island NY). To reduce any shock to cells and maintain them under physiological conditions, the medium was warmed to 37 °C.

Mitochondria, Endoplasmic Reticulum and Golgi Apparatus labeling.

For locating the cellular organelles, we utilized designated probes from Thermo Fisher Scientific. We applied MitoTracker Green FM (Life Technologies Corporation, Grand Island NY) for labelling the mitochondria, ER-Tracker Green (Life Technologies Corporation, Grand Island NY) for labeling of Endoplasmic Reticulum, and NBD C6 ceramide-BSA (Life Technologies Corporation, Grand Island NY) for labeling of Golgi Apparatus in accordance with the manufacturer’s instructions after 12–36 h or more to allow for sufficient cellular adhesion to the glass bottom (50% confluency). Using other fluorescent reporters for organelle labeling may affect the BCA accuracy due to the distinction of Raman profiles of other dyes from those used in background subtraction software. Following labeling, the cells were washed by warmed, sterile PBS three times, and then the optically transparent DMEM supplemented with 25 mM of HEPES was added.

RESULTS AND DISCUSSION

Single organelle lipidome parameters extracted by BCAbox.

Representative Raman spectra of lipids in Apparatus Golgi (AG), Endoplasmic Reticulum (ER), Mitochondria and Lipid Droplets (LD) of live HeLa cells obtained by BCAbox are

shown in Figure 2. Lipid spectra in mitochondria, ER, and AG are slightly “noisier” (Figure 2a) compared to those of lipid droplets (Figure 2b) due to a lower concentration of phospholipids in these organelles.

Phospholipid concentration.—The intensity of the peak at 1443 cm^{-1} in lipid spectrum, which is assigned to bending of all CH_2 and CH_3 molecular bonds, is used for phospholipid concentration measurement. Calibration of DXR2 Raman microscope, using a concentration set of bovine brain lipids (Avanti lipids) solution in chloroform, resulted in 25.3 cps (counts per second) Raman intensity of this peak for $\sim 14\text{ mg/ml}$.

Unsaturation level of lipids.—Areas of two specific spectral bands at 1655 cm^{-1} , assigned to the $\text{C}=\text{C}$ stretching mode, proportional to the amount of unsaturated $\text{C}=\text{C}$ bonds, and at 1443 cm^{-1} , assigned to CH_2 scissoring/bending modes, which is proportional to the amount of saturated $\text{C}-\text{C}$ bonds, (Figure S4) were used for estimation of the unsaturation level of lipids (parameter LSU as a ratio of the corresponding areas³⁴). LSU is close to zero for saturated palmitic acid, 0.52 for the monounsaturated oleic acid and 0.93 for the polyunsaturated linoleic acid (Figure S5). Insets in Figure 2 show that the unsaturation level of lipids in live HeLa cells can be significantly varying in different probed organelles.

Trans-/Cis- ratio.—The ratio of the intensities at 1666 cm^{-1} and 1655 cm^{-1} represents the ratio of trans- to cis- carbon-carbon bond amount in lipid species, and can be used as a quantitative parameter (TCP) for isomers ratio of lipid molecules³⁴. For oleic acid and linoleic acids with only cis- conformation of unsaturated $\text{C}=\text{C}$ bonds, TCP is equal to 0.45 (Figure S5), while for phospholipids with one and two trans- $\text{C}=\text{C}$ bonds, the corresponding parameters are 0.92 and 1.71 respectively.

Sphingolipids and Cholesterol content.—The presence of cholesterol and sphingolipids in probed organelle can be estimated by the intensity of their corresponding characteristic peaks at $\sim 700\text{ cm}^{-1}$ and 717 cm^{-1} , respectively. For pure egg sphingomyelin and N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine (both from Avanti lipids), the PC/SM parameter (sphingolipids content, 717 cm^{-1}) is equal to ~ 1.00 . The same value of the cholesterol content (CLA parameter), estimated by 700 cm^{-1} peak intensity, i.e. $\text{CLA}=1$ is resulted from the measured spectra of pure cholesteryl linoleate (Figure S6).

Organellar lipidome analysis.

In this study, we aimed to identify the lipid composition, and characterize its natural variations in major cellular organelles of live cells. Raman spectra were acquired in several cell lines from ER, AG, and mitochondria located by organelle-specific stains, as described above (Figure 1). In addition, the spectra were acquired from the lipid droplets, which were locate by transmitted light imaging. The spectra were processed, contributions of other than lipids molecules were subtracted, and the lipid profiles were further analyzed. To illustrate the variations between different organelles, scattered graphs were generated with identifiable lipid parameters plotted against the total lipid content. Data obtained in different cell lines are shown in Figures 3 and 4, where each point on charts represents a single organelle measurement. The graphs demonstrate that phospholipids in HeLa cells contain saturated,

mono- and polyunsaturated components, as well as sphingolipid species. Cloud distributions on the graphs show, that in spite of the parameter heterogeneity, the difference of lipidome parameters between organelles of the same cell line, as well as between the same organelles of different cell lines, is clearly observed.

Heterogeneity of organellar lipidomes in cultured cells.—Compartmentalization of biochemical processes suggests formation of different molecular environment in each type of cellular organelle. To gain insight into organelle-specific lipid content, we compared lipidomes in ER, AG, mitochondria and LD in HeLa cells. Consistent with different functions of these organelles in cellular metabolism, we report significant differences in the lipid composition. A one-way analysis of variance (Table S1) shows that AG and LD contain at least 20% less of mono- and polyunsaturated lipids ($LSU_{AG} = 0.29$) compared to that in ER ($LSU_{ER} = 0.35$) and mitochondria ($LSU_{mito} = 0.37$). This level of unsaturation is close to that of phospholipids with two chains of monounsaturated fatty acids (horizontal line 2 in Figure 3a). Also, LDs of HeLa cells contain less phospholipids with trans- C=C conformations ($TCP_{LD} = 0.44$), than in other organelles ($TCP_{AG} = 0.56$, $TCP_{ER} = 0.58$, $TCP_{mito} = 0.64$). This TCP parameter demonstrates that the trans- part of phospholipids in HeLa lipid droplets is close to zero. Besides, lipid droplets of HeLa cells contain more than 5 times less sphingolipids ($PC/SM_{LD} = 0.12$) compared to others organelles ($PC/SM_{AG} = 0.63$, $PC/SM_{ER} = 0.69$, $PC/SM_{mito} = 0.70$).

Interline comparison of organellar lipidome.—In this section, we compared lipidome of two organelles, AG and LD, for three cell lines – HeLa, human malignant glioblastoma cells (U251) and human brain vascular endothelial cells (BMEC). Lipid droplets accumulation represents a biomarker of many cancers^{35, 36}, and is specifically correlated with increased tumor progression in glioma. Herein, using our method, we can go a step further to compare the composition of lipids in LDs with other organelles such as AG, with the goal to obtain information on the dynamics between lipid trafficking inside live cells in real time. In all the cell lines that we measured, we noticed a drastic difference between the saturation of lipids in Golgi apparatus as compared to other cellular sites. As seen from Figure 4 and Table S2, the number of unsaturated lipids in the AG membranes of HeLa cells ($LSU = 0.29$), is more than 1.5 times lower than that in both BMEC (0.46) and U251 cell lines (0.50). The content of unsaturated phospholipids in lipid droplets is the same as that in AG of HeLa cells and, again, lower than that in lipid droplets for U251 ($LSU = 0.43$) and BMEC ($LSU = 0.37$). Furthermore, our platform identified that even the same organelles of these cell lines differ significantly in their lipid content (Figure 4). From all the three lines, HeLa cells contain fewer phospholipids with trans- C=C conformations, than other cells, which is most likely due to a lower content of unsaturated phospholipids in HeLa ER and LD organelles (Figure 4b). At the same time, the sphingolipids concentrations in AG of HeLa and U251 are very close (PC/SM 0.63 and 0.69), and slightly lower than that in AG of BMEC (0.73) (Figure 4c). The largest difference of sphingolipids content in all these three lines are found in lipid droplets. In the BMEC line, it is close to zero (0.06); in LD of the U251 cell line, this peak achieves 0.34, while HeLa sphingolipids in LD are in between (0.12) of that for BMEC and U251. This shows that our method is sensitive enough to

differentiate the lipid profiles of organelles between different cell, and has the potential to quantify changes as a function of different treatments.

Sensitivity of organellar lipidome to drug intervention.—A potential strength of the Raman lipidomic platform, is the capability to monitor cellular response to different drugs at the organelle level³⁷. As an example of this application, we employed U251 glioma cells, which were engineered to express an isocitrate dehydrogenase 1 (IDH) mutation, R132C. This mutation is one of the early events of gliomagenesis and is very prevalent in lower grade gliomas; it is a major target for pharmaceutical intervention^{38–41}. To test whether the organellar lipidome can be used as a biomarker of drug response, we treated the IDH mutated gliomas with the most utilized inhibitor (AGI 5198) that acts to prevent D-2hydroxyglutarate synthesis, and therefore corrects the defect induced by mutation⁴⁰. We found that organellar lipidome profile is drastically changed in the endoplasmic reticulum in presence of the inhibitor. Four different parameters used here describe the changes in the organellar lipidome: 1) Total lipid content and LSU decreased upon drug treatment, reflecting an enrichment of saturated fatty acids in endoplasmic reticulum (Figure 5); 2) The sphingolipids content and lipid trans isomers also decreased in lipid droplets in the presence of the inhibitor, suggesting a potential re-distribution of these lipids from storage into circulation to restore lipid homeostasis. We find that this organelle Raman lipidomics approach can report on the effect of drugs at the organelle level, creating a major opportunity for future drug screens.

Analysis of measured data shows that a detectable amount of cholesterol in probed organelles, estimated by the presence of a characteristic peak at $\sim 700\text{ cm}^{-1}$ is observed only in $\sim 15\%$ cases of measurements (Figure S7). These data indicate a low amount of this species in cultured cell lines chosen for our experiment. Due to this fact, we did not include these results in our analysis. Most likely, a larger measurement statistics is necessary to achieve any reliable analysis of cholesterol content in cultured cells.

CONCLUSION

We introduce here a micro Raman lipidomics platform with a unique capability to analyze microscopic organelles in live cultured cells. A key component of this platform is BCABox - a first software that interrogates distinct subclasses of lipids in the subcellular structures. Using micro Raman platform, total lipids concentration, as well as fractions of sphingolipids and cholesterol, the lipids unsaturation levels and trans/cis ratio for unsaturated carbon bonds could be readily quantified in each studied organelle. While mass-spectrometry provides with unsurpassed chemical selectivity recognizing close to 1000 species of fatty acids, the microRaman-BCA platform has significantly higher mass detection sensitivity, which permits to analyze single organelles. Furthermore, the noninvasive nature of Raman techniques enables to monitor organelles lipidomes in real time. Thus, presented here platform can significantly expand an arsenal of lipidomics tools. A single-organelle non-destructive analysis is especially important for spatiotemporal studies of cellular metabolism, heterogeneity, differentiation, and abnormal transformations. Furthermore, this tool could unravel organelle-specific biomarkers to provide an insight into molecular mechanisms of disease development, aid diagnosis and monitor progress in medical

treatment. The BCAbox , together with developed protocols , will help to standardize micro-Raman data for bio-applications and facilitate data cross-analysis among different research groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overview of Raman-based lipids quantification at the organelle level.

In Steps 1 and 2, diverse subcellular structures are labeled by specific fluorescence probes and located by high-resolution microscopy. In Steps 3 and 4, Raman spectra are collected from the labeled organelles, and BCABox algorithm is applied to extract lipid profiles. In the last step, the lipid composition is analyzed; as an example, the chart shows levels of lipids unsaturation parameter plotted against the absolute weight of lipids in apparatus Golgi, endoplasmic reticulum or mitochondria of HeLa cells, as indicated. The unsaturation of lipids (LSU) is computed based upon the area ratio of 1655 cm^{-1} to 1443 cm^{-1} . Via the same procedure, the TCP parameter, which characterizes trans/cis C=C bonds ratio in lipid species can be obtained from the intensities at 1666 cm^{-1} and 1655 cm^{-1} . Abbreviations: AG – Apparatus Golgi, ER – Endoplasmic Reticulum, mito– mitochondrion, LD – lipid droplet, LSU – lipids unsaturation parameter, TCP trans-/cis– parameter.

Figure 2. Raman spectra of organellar lipids.

Raman profiles of organellar lipids in a live HeLa normalized to the peak at 1443 cm^{-1} . Panel (a) represents lipid profiles of Apparatus Golgi (AG), Endoplasmic Reticulum (ER) and mitochondria (mito); panel (b) shows lipid profiles in lipid droplets (LD); numbers indicate order of cell in database. Insets show a part of the spectra zoomed in the range of 1400 and 1700 cm^{-1} . This example illustrates the diversity of the unsaturation level of lipids in live HeLa cells grown in the same dish, by different intensities of the peak at 1665 cm^{-1} for normalization at 1443 cm^{-1} .

Figure 3. Organellar lipidome parameters (LSU, TCP, PC/SM) and lipid concentration in the organelles of HeLa cells.

Concentration of lipids vs corresponding LSU as a merit of unsaturation of lipids (a), TCP as a merit of trans- to cis- ratio of carbon-carbon bonds of cellular lipids (b), and the sphingolipids content PC/SM (c) probed in different organelles of HeLa cells in the same dish. The standard error is comparable to the size of symbols. Abbreviations: AG – apparatus Golgi, ER – endoplasmic reticulum, mito – mitochondrion, LD – lipid droplets. The levels of LSU shown by numbered horizontal lines in graph (a) correspond to: (1) Egg SM (16:0/18:1), LSU=0.19; (2) 16:1 SM (16:1/18:1), LSU=0.29; (3) OA (18:1), LSU=0.52. Levels of TCP shown by numbered horizontal lines in graph (b) correspond to: (1):OA (18:1E), LA (18:2E), TCP~0.45; (2) 16:1 SM (16:1E/18:1Z), TCP=0.92 (3) Egg SM (16:0/18:1Z), TCP=1.71.

OA is oleic acid, LA is linoleic acid, Egg SM is chicken egg sphingomyelin and 16:1 SM is N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine. The numbers in parenthesis show the number of carbon bonds (first) and the number of double bonds (second); the letter after the second number denotes cis- (E) or trans- (Z) isomer of the double bond.

Figure 4. Organellar lipidome parameters LSU, TCP, PC/SM and lipid concentration of different cell lines.

Concentration of organellar lipids vs corresponding LSU (a) TCP (b), and PC/SM (c) of lipid constituents in AG (solid circles) and lipid droplets (open circles) for three cell lines – HeLa (red), U251 (human malignant glioblastoma cell line, black) and BMEC (human brain vascular endothelial cells, green). The standard error is comparable to the size of symbols. Abbreviations: AG – apparatus Golgi, ER – endoplasmic reticulum, mito – mitochondrion, LD – lipid droplets.

As a reference, the levels of LSU shown by numbered horizontal lines in graph (a) correspond to: (1) Egg SM (16:0/18:1), (2) 16:1 SM (16:1/18:1), (3) OA (18:1). Levels of TCP shown by numbered horizontal lines in graph (b) correspond to: (1):OA (18:1E), LA (18:2E), (2) 16:1 SM (16:1E/18:1Z) (3) Egg SM (16:0/18:1Z).

OA is oleic acid, LA is linoleic acid, Egg SM is chicken egg sphingomyelin, 16:1 SM is N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine. Numbers in parenthesis show the number of carbon bonds (first) and the number of double bonds (second); letter after the second number denotes cis- (E) or trans- (Z) isomer of the double bond.

Figure 5. Unsaturation parameter (LSU) of lipids in AG of U251R132C cells before and after 48 hours of application of AGI5198 inhibitor.

A significant difference of LSU was found after drug application (Table S3). Abbreviations: ER – endoplasmic reticulum. As reference, the levels of LSU shown by numbered horizontal lines in graph (a) correspond to: (2) 16:1 SM (16:1/18:1), (3) OA (18:1). Where OA is oleic acid, 16:1 SM is N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine. Numbers in parenthesis show the number of carbon bonds (first) and the number of double bonds (second); letter after the second number denotes cis- (E) or trans- (Z) isomer of the double bond.