博　士　論　文　（要　約）

Development of Raman microscopy-based analytical methods for intracellular localization of labeled fatty acids and physical properties of lipid environment

（ラマン顕微鏡による標識脂肪酸の細胞内局在および脂質物性の解析技術開発）

植　松　真　章

論文の内容の要旨

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Introduction

Lipids are the fundamental components of all living things forming boundaries of cells and organelles, and their physical properties such as viscosity are homeostatically maintained inside cells. Physical properties of cellular membranes are mainly regulated by tuning the acyl chain composition of phospholipids. In general, the higher degree of unsaturation of acyl chains increases membrane flexibility. They are also involved in physiological roles such as enzymatic activities and signal induction through regulating the diffusion speed of membrane proteins or rigidity of membranes. Therefore, the visualization of multiple lipid species and the measurement of physical properties of lipids inside cells are very crucial to understand how lipids are involved in biological roles.

Various probes and methods have been developed to measure subcellular localization of lipids. Chemical or genetically-encoded lipid probes against phosphatidylserine, phosphatidylinositol phosphate, ceramide, and so on are commonly used, allowing us to visualize the distribution of lipid species based on their polar head groups. Attempts have also been made to visualize the localization of fatty acyl groups inside in the hydrophobic tail of the lipid by introducing the fluorophore-conjugated lipids. However, the sizes of the lipid molecules are too small to label with fluorescent tags without changing their original characteristics. In addition, most of the acyl groups are usually buried inside lipid bilayers or lipid droplets (LDs) in the form of phospholipids, triacylglycerols (TAG), cholesterol esters, and so on. As a result, even probes targeting fatty acyl chains inside those lipids cannot reach them, and little is known about their intracellular distribution and stimulus-dependent mobilization.

As for the quantification of the physical properties of biological membranes at subcellular level, the use of exogenously introduced or genetically-encoded fluorescent probes are commonly used. However, these methods involve the artificial effect of probes themselves on the physical properties of membranes being analyzed. In addition to this, most of the probes are mainly targeting at plasma membranes, and other

intracellular structures were yet to be analyzed. Especially, physical properties of LDs, which are mainly composed of TAG, were hardly quantified nor studied due to the lack of appropriate probes, even though nearly all eukaryotic cells can make LDs. Thus, it is unclear how LDs contribute to the physical properties of cellular lipid environment were unknown, and had been thought that they simply store extra energy like excess fatty acids in the form of triglyceride or cholesterol esters.

One of the promising solutions for these problems is to use Raman microscopy, which can detect the vibrational frequencies of chemical bonds of molecules. Deuterium-labeled fatty acids were sometimes observed by Raman microscopy to track their uptake into cells, since the carbon-deuterium (C–D) chemical bond does not exist in biological samples and its Raman spectra can be distinguished from those of endogenous biomolecules. However, multiplex imaging of small compounds like lipids without tagging large molecules were yet to be achieved, hindering the analysis of behavioral differences of lipids. For the measurement of the physical properties of lipids, previous in vitro studies have demonstrated that Raman spectra of carbon-hydrogen (C–H) stretch from fatty acids or phospholipids show slightly different spectra depending on the viscosity associated with extreme changes in temperature or pressure. However, it has been unknown whether the spectrum of C–H stretch is sensitive enough to differentiate physical properties of lipids under physiological in vivo conditions. It is also necessary to developed analytical methods to differentiate the spectra of pure from those of biomolecules and to convert the observed spectra into quantitative value of the physical properties of lipids.

Results

In this study, I developed two Raman microscopy-based approaches under deuterium-labeled fatty acid treatment; one is to visualize up to five atomically-labeled fatty acids, and the other is to quantify physical properties of subcellular lipids as the gauche/trans conformational ratio, which can be used as an indicator of viscosity.

For the first method, fatty acids labeled with various atoms were prepared to enable the observation of as many fatty acid species as possible while minimizing the labeling effect on lipids. In addition, the shapes of the Raman spectra were found to be different depending on the labeling patterns for the deuterium-labeled fatty acids. These findings suggested that observation of up to five fatty acids species can be observed at the same time. However, although these spectra showed characteristic shapes, some of them overlapped with each other, making it difficult to analyze them accurately. To solve this problem, I created an open-source software, ImageCUBE, that

can automatically separate overlapping spectra from the mixed measured spectrum. Briefly, I hypothesized that the measured spectrum is a mixture of labeled fatty acid spectra, a background spectrum, and a baseline drift, then the coefficients of each component were calculated by making the difference between the sum of the unmixed and measured spectra as small as possible.

By using this developed analytical procedure, I observed how exogenous fatty acids are taken up into HeLa cells and are distributed inside them. As a result, despite the differences in molecular structures and functions between fatty acid species, their distributions were found to be very similar, that is, they formed punctate structures at almost exactly the same positions and weak signals were also observed in other cytoplasmic area. After confirming that the accumulated punctate structures are LDs, the differences in the composition of the exogenous labeled fatty acids in LD and non-LD regions were compared. The results revealed that fatty acids with larger degrees of unsaturation were more concentrated in LD areas than in non-LD areas.

For the second method, the quantification of physical properties of lipids, I used target lipid itself as a probe to overcome the innate problems of conventional probes affecting the lipid environment. I found that Raman spectra of carbon-deuterium (C–D) stretch from deuterium labeled saturated fatty acids were altered by the surrounding lipid environment in vitro. The results indicate that these fatty acids can be used as a probe to measure the physical properties of lipid environment. In order to reveal what kind of physical properties of lipid environment affect this spectral transition, I performed in silico simulation of Raman spectra using molecules in different conformational states. As a result, differences in gauche/trans conformational states of fatty acids qualitatively reproduced the in vitro spectra. Then, based on the experimentally measured reference spectral transitions in vitro and model membrane simulations in silico, the analytical method was developed to quantitatively convert the observed spectra into the gauche/trans ratio of labeled fatty acids.

Since the incorporated labeled fatty acids localized on LDs, investigation of the physical properties of LDs became possible. Application of this quantification method to HeLa cells treated with various combination of fatty acids and pharmacological interventions revealed that the while the gauche/trans ratio in LDs dynamically changed, that in non-LD regions were relatively constant.

Discussion

Utilization of distinctive spectra patterns of atomically-labeled fatty acids and unmixing algorithm to separate overlapped spectra enabled us to perform multiplex

imaging of fatty acids. Previous localizations of intracellular fatty acids mainly involved biochemical procedures, which have poor spatial resolution, making it difficult to uncover specific subcellular distributions. The biological application of this method revealed the differences in the behavior of incorporated fatty acids in HeLa cells; that is, the incorporated fatty acids with higher degrees of unsaturation are more efficiently concentrated in LDs. The biological mechanism of this phenomenon remains unclear, however. Substrate preferences of enzymes involving in the synthesis and the degradation of LDs might be involved. Regulation of fatty acid composition in membrane phospholipid, major fatty acid storage in non-LD area, might also cause this heterogenous fatty acid distributions, possibly through de novo and remodeling pathways of phospholipids. The biological significance of the distribution differences of fatty acid is another interesting question. Considering the property that PUFAs are easily oxidized, LDs may serve as shelters to protect them from being oxidized inside cells.

As for the quantification of the physical properties of lipids, the fundamental difference of the developed method to the conventional approaches is that I use lipid itself as a probe. This simple ingenuity enables us to directly measure the physical properties of lipid while preventing them being disturbed by the introduction of probes. Although my technique is limited to the fatty acid treated conditions, such stimuli are commonly used to study intracellular lipid transportation and the function of LDs, and should allow us to study them under more natural conditions than other existing techniques.

The quantification of physical properties of lipid environment revealed that the gauche/trans ratio of LDs changed dramatically in response to the fatty acid treatment or pharmacological intervention, while that of non-LD regions were relatively constant. These results imply the existence of some mechanisms to maintain the cytoplasmic lipid environment, perhaps with LDs acting as a buffer for excessive "gauche/trans ratio". This new biological phenomenon gave new viewpoint of the functions of LDs, which were previously just regarded as the energy storage organelle. The signal of incorporated fatty acids in non-LD region can be regarded mainly as ER membranes, and robustness of the physical properties of the ER membrane may be important to maintain the normal cell functions, such as synthesis, processing, and transport of proteins.

The developed methods will contribute to the understanding of the biological functions and regulatory mechanisms of spatial dynamics of fatty acids and the physical state of intracellular lipid environments.