Function and Structure Analysis of Glycolipid Microdomains

Kazuya Kabayama
Graduate School of Science, Osaka University, Machikaneyama 1–1, Toyonaka, Osaka 560–0043, Japan
FAX: +81–6–6850–5419, E-mail: kaba@chem.sci.osaka-u.ac.jp
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Abstract
Glycosphingolipids are not uniformly present in cell membranes and form microdomains called glycolipid microdomains together with sphingomyelin and cholesterol. Many signaling molecules accumulate there, and glycolipid composition greatly influences signal transmission efficiency. Therefore, analyzing the dynamics and structure of glycolipid microdomains is very important in understanding life phenomena. In recent glycolipid microdomain studies, along with biochemical methods, analysis of membrane molecular dynamics by fluorescence microscopy, localization analysis using an electron microscope, and lipid structure analysis by mass spectrometry have been conducted. In this review, I mainly introduce the glycolipid microdomain analysis method using fluorescence microscopy and mass spectrometry.

A. Introduction
Previous studies have demonstrated that membrane heterogeneity plays an important role in biological membranes, and the concept of lipid rafts is fundamental to this heterogeneity. However, in the field of lipid raft research, analytical methods are at a standstill, and physical methods of studying the biological membrane as a liquid layer dominated by the spatiotemporal space are still under development. This creates a large void between biological and physicochemical techniques in analyzing actual cells. Lipid rafts accumulate a lot of glycosphingolipids, so lipid rafts are specifically referred to as glycolipid microdomains in this review. On the other hand, there are also sterol non-containing microdomain (1), or saturated fatty acid-containing glyceroglycolipids exist as a glycolipid microdomains (2, 3). Gangliosides, which are known as a constituent of glycolipid microdomains, is a generic term for the glycosphingolipid family including sialic acid, and GM3 is the first molecule in its biosynthetic pathway. Since GM3 is widely expressed in various cells of mammals including humans, physiological functions and pathophysiologically significance are becoming of interest. Gangliosides are expressed on the cell membrane surface and are involved in adhesion and infiltration of cancer cells, receptor activity, growth factor-like activity and intercellular recognition. There are many reports of their involvement in relation to the malignancy of cancer. As one of the mechanisms of these actions, accumulation or dissociation of membrane receptor molecules into the glycolipid microdomains is proposed. However, at present, the analytical method for glycolipid microdomains is limited to a technique of evaluating their heterogeneous localization on the cell membrane by whether it is insoluble or soluble in the detergent as mainly determined with sucrose density gradient centrifugation. No new procedure have been developed beyond this. Furthermore, nonionic detergents such as the commonly used Triton X-100 have the property of reducing lipid–protein interactions. Evaluating the accumulation or dissociation of membrane receptor molecules in glycolipid microdomains becomes difficult as electrostatic interactions must be taken into consideration. Therefore, we intended to elucidate the function and structure of the glycolipid microdomain with a methods to eliminate detergents thoroughly, or without using detergents. For the former, we considered introducing molecular dynamics analysis using a fluorescence microscope. In recent years, there has been remarkable development of visualization technology using fluorescent labeled molecules, and molecular dynamics analysis is gaining attention not only for its use in basic research but also for clinical applications. For the latter method, a convenient separation technique for detergent removal using an organic solvent, which is also useful for pretreatment in mass spectrometry, has been developed. Each method will be outlined from the viewpoint of its utility to evaluate glycolipid microdomains.

B. Difficulties of Glycolipid Microdomain Analysis
At present, several problems are encountered in analyzing the function and structure of glycolipid microdomains. First, membrane lipids are not fixed in sample treatment for analysis by general electron microscopes. Nevertheless, immunoelectron microscopy of glycolipids has been carried out using samples immobilized with glutaraldehyde. There are many reports to discuss that accumulation of colloidal gold due to aggregation properties of anti-glycolipid antibodies (mainly IgM antibodies) is a lipid raft structure. To resolve this issue, Fujimoto et al. have adopted a method of completely fixing lipids by a rapid freezing method (4). Further, Kusumi et al. used total internal reflection fluorescence microscopy to analyze the dynamics of lipid and lipid-anchored
proteins in detail; they demonstrated that membrane lipids cannot be immobilized, and called for reevaluating of marking data with conventional antibodies (5). Secondly, it is difficult to comprehensively interpret glycolipid microdomains only via fractionation of lipid rafts by density gradient centrifugation. In other words, because the molecules accumulated depend on the concentration and type of detergent, it is not possible to obtain a unified view (6). Regarding this issue, many discussions have taken place so far, but no conclusions have been drawn. As a potential solution to the problem, in addition to biochemical data, we used live cell imaging with a fluorescence microscope to elucidate the actual glycolipid microdomains. Thirdly, the detergent in the membrane microdomain fraction interferes with Time-of-Flight Mass Spectrometer (TOF-MS) analysis. To address the issue, this article describes the use of a triple quadrupole mass spectrometer (Triple-Q TOF-MS) that is recently becoming widely operated, and the separation of glycolipids from the detergent using dichloroethane.

C. Molecular Dynamics Analysis Using Fluorescence Microscopy

Currently, the optical microscope is shifting to coexistence of “observing” and “measuring” capabilities. That is, it is used as an analytical instrument for visualizing and analyzing the expression, dynamics, and functions of molecules. Optical-based microscopy technologies have also taken an important position as an analytical technique in recent years. As an example, various microscopy technologies have been awarded at the annual “Method of the Year” (7) which the publication Nature Methods started in 2007 to honor analytical techniques. Selected technologies include super-resolution fluorescence microscopy in 2008, optogenetics in 2010, light-sheet fluorescence microscopy in 2014, and single particle cryo-electron microscopy in 2015, indicating that microscopy methods are useful and indispensable. In particular, the inventors of the super-resolution microscope and the cryo-electron microscope garnered the Nobel Prize Award several years later, respectively. We believe that microscopy techniques are a very useful tool for elucidating cell biological phenomena and have previously reported on lipid rafts using confocal laser scanning microscopes and electron microscopes (8, 9). In the following sections, we describe the function and structure analysis method of glycolipid microdomains using microscopy and mass spectrometry technologies.

D. Glycolipid Microdomain Analysis Using Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP) is used as a molecular dynamics analysis technique using fluorescence microscopy, especially for measuring fluidity of cell membrane molecules (10, 11). In FRAP, it is necessary to apply a fluorescent label to the target molecule. In a living cell in which the labeled molecule is expressed, the region to be analyzed is briefly irradiated with a strong laser to fade only the fluorescence without destroying the function of the molecule. If the target molecule is immobile, the fluorescence in the faded region will not be recovered. If the target molecule is mobile, it diffuses and mixes with surrounding non-faded molecules, and the fluorescence in the faded region recovers. By plotting the time course of fluorescence recovery rate

Fig. 1. Graph showing time course of fluorescence recovery rate and outline of fluorescence recovery method after photobleaching (FRAP): The dynamic component ratio of the target molecule can be obtained from the terminal point of the recovery curve (predictable by nonlinear curve fitting). It is also possible to calculate the diffusion rate from the rising speed of the curve (A). Example of FRAP: GFP fusion insulin receptor (IR) (IR-GFP) and caveolin 1 (Cav1) (Cav1-GFP) were expressed in HeLa cells, and the membrane surface was observed time-sequentially by confocal microscopy (Top and bottom images, respectively). ROI (measurement area) was bleached 16s after the start of measurement, and images after 1min and 3min were acquired. Since IR diffuses on the cell membrane, the fluorescence intensity of the ROI recovers with time. On the other hand, since Cav1 forms a caveolae structure and is immobilized, the fluorescence luminance does not recover (B).
on a graph, it is possible to calculate the dynamic component ratio and the diffusion rate of the target molecule. An example of FRAP is shown in Fig. 1. With this method, changes in membrane fluidity due to changes in composition of constituents of glycolipid microdomains are analyzed. By combining this information with results obtained from the biochemically isolated, detergent-resistant microdomain (DRM), it is possible to analyze and compare the dynamics of lipid raft environments on the actual cell membrane. Simons et al., who originally proposed lipid rafts, have performed FRAP analysis of various membrane proteins expressed on apical membranes of MDCK cells to demonstrate the existence of domains that phase separate in biological membranes (12). By using this method, we also succeeded in analyzing the electrostatic interaction between acidic glycolipid and membrane receptor proteins accumulating in the glycolipid microdomain (8).

E. Glycolipid Microdomain Analysis Using Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) is a method that can measure the diffusion coefficient, molecular weight, and even the size of target molecule by replacing fluctuation of photons emitted by fluorescent molecules passing through the focal region of the microscope with a correlation function (13). In relation to carbohydrate substances, Mizuno and Inazu et al. reported the evaluation of binding between fluorescence-labeled oligosaccharides and lectin by FCS in an in vitro system (14). Recently, Hell et al. developed a method to analyze detailed membrane lipid dynamics in living cells using FCS measurements and a super-resolution microscope (15). However, since the measurement principle is difficult to understand, and the detection system is expensive, the method is currently not widely used. In addition, in the field of biology, they may consider that there are interactions among molecules to be more important than how fast are the molecules moving. Therefore, in recent years, an analysis method that has been emerging is fluorescence cross correlation spectroscopy (FCCS), which analyzes the simultaneity of fluorescent molecules of two colors. Regarding the principles and detailed methods of FCS and FCCS, there are excellent reviews (16) by experts, thus we will omit them here and briefly describe only the concepts in this article. When separate fluorescent molecules (or fluorescently-labeled molecules) do not interact, the molecules pass through the focal region of the microscope at different time points. In this case, since the fluorescence emitted from the molecules do not have simultaneity in the time axis, the cross correlation function does not arise (Fig. 2A). On the other hand, when two fluorescent molecules interact with each other, they simultaneously pass through the focal region, and the cross correlation function increases (Fig. 2B). From the comparison of these values, the presence or absence of interaction is determined. An example of FCCS using ganglioside GM1 and cholera toxin B subunit is shown (Fig. 2C). Although dedicated instruments specialized for FCCS analysis are commercially available, measurements also can be conducted by simply adding a highly sensitive electron multiplier called APD (Avalanche photodiode) to an existing confocal microscope. We believe that this analytical method is applicable to analyze carbohydrate chain-lectin interactions in glycolipid microdomains. In addition, Gratton

![Fig. 2. Outline of Fluorescence Cross Correlation Spectroscopy (FCCS): When the two fluorescent molecules do not interact, the fluorescence emitted by the molecules do not have simultaneity, and the cross correlation function (black line) does not rise (A). In contrast, when two fluorescent molecules interact with each other, the cross correlation function rises at the same time because they simultaneously passes through the focal region (B). Example of interaction analysis by FCCS: FCCS analysis results from four types of sphingolipids (sphingomyelin (SM), glucosylceramide (GlcCer), lactosylceramide (LacCer), and ganglioside (GM1) labeled with BODIPY and cholera toxin B subunit (CTX) labeled with Alexa Fluor® 647 are shown. Cross correlation coefficient with CTX-Alexa 647 selectively increased only with BODIPY-GM1 (C).](image-url)
F. Glycolipid Microdomain Analysis Using Single Particle Tracking

Single particle tracking analysis (SPT) is also a useful technique for analyzing lateral diffusion of membrane receptors. To visualize one particle on the cell membrane, a total internal reflection fluorescence microscope is used (19, 20). The principles behind this technique are noted in a prior review (21). There are research reports using artificial membranes in the fields of biophysics and surface science which mentioned that this method can be applied to analysis of the lipid rafts (22, 23). Detailed analysis of lipid rafts in living cells has also been conducted in recent years (24). To analyze the dynamics of the glycolipid microdomain, it is desirable to use a probe that does not fluorescently label the lipid portion so as not to affect the phase separation state of the membrane. Recently, Kiso and Kusumi et al. have reported a system that performs fluorescence labeling on the non-reducing terminal part of the ganglioside glycan chain and single particle tracking analysis (25). Previously, using a total internal reflection fluorescence microscope, we also conducted dynamic observations of the insulin receptor (IR-GFP) and caveolin-1 (Cav1-GFP) fused with fluorescent proteins on the cell membrane (8). However, when performing diffusion measurements using a total internal reflection microscope, fluorescent proteins such as GFP are prone to fade rapidly (fading is completed about 5 s). Since restrictions are imposed on the setting of conditions, it is therefore necessary to use fluorescent molecules with high photostability. Thus, we prepared IR-TMR and Cav1-TMR labeled with tetramethylrhodamine (TMR) using HaloTag technology (Promega) in which a tag protein forms a specific covalent bond with a small molecule ligand, and conducted an analysis. As a result, it was possible to observe one particle (although verification is still necessary to determine whether it is one molecule) of the labeled molecules for about 15 s. By measuring the diffusion coefficients of approximately 100 particles, it was possible to display the dynamics of the target molecules in a histogram. That is, since lateral diffusion of membrane molecules can be individually verified, information that cannot be extracted from the ensemble average can be extracted in a heterogeneous membrane environment in which a glycolipid microdomain exists. For measurements of diffusion coefficients, luminescent spot tracking can facilitate the progression from image acquisition to analysis. Tracking can be performed by using a plugin introduced to the free analysis application Image-J or by employing a commercially available application such as G-Track (G-angstrom Co.) (Fig. 3).

G. Simple Method for Complete Detergent Removal from Lipid Raft Fractions

In addition to the dynamic analysis of cell membrane molecules by fluorescence microscopy, structural analysis of glycolipids accumulated in lipid rafts is also an important means for understanding the principles of glycolipid microdomain construction. As a biochemical technique for separating and purifying lipid raft molecules, a method of recovering detergent- resistant microdomains (DRM) by sucrose density gradient centrifugation at low temperature is generally used. Nonionic detergents such as Triton X-100, Lubrol 98, Brij 58/97, and NP-40 are commonly used in this procedure (26, 27). Since detergents are very similar in structure and physical properties to glycolipids, thorough elimination of detergents is essential in mass spectrometry of glycolipids contained in DRM. Nevertheless, the fact that a convenient method for separating glycolipids from detergents has not been found has
hindered analysis of the glycolipid microdomain components (Fig. 4A, B). Therefore, we performed method development by drying until solid, a mixture of GM3 and Triton X-100 in test tubes, then washing the solids with 18 different kinds of organic solvents. The components of the fractions in the organic solvents and that remaining in the test tubes were compared. As a result, we succeeded in completely fractionating the two components by using dichloroethane (DCE) (28). Based on this finding, spectral measurements using MALDI-QIT-TOF MS were carried out with DRM after the Svennerholm procedure and DCE washing. No detergent-derived peaks were detected in the DRM and we succeeded in identifying GM3 molecular species with different fatty acid chain lengths (Fig. 4C). By this method, structural analysis of glycolipids localized in lipid rafts as well as glycolipid composition of whole cells or tissues became possible. Therefore, it is expected that reports on comparative analysis related to pathological conditions and the like will increase in the future.

H. Perspective
Recent fluorescence microscope technologies have embarked on a new trend to explore the principles of super-resolution and harness them into practical use. On the other hand, by improving existing equipment or applications, precise measuring instruments can be developed. In the analysis using the FRAP method, it is possible to separate dynamic molecules into different velocity components by performing nonlinear curve fitting on the fluorescence recovery curve, from which qualitative changes in the membrane can be predicted. Recently, Image Correlation Spectroscopy (ICS), which extended the concept of FCS to two dimensions, has been used for analysis of membrane molecules (29, 30). We are considering whether we can elucidate the function of glycolipid microdomains by using the Raster Image Correlation Spectroscopy (RICS) (18, 31). This allows for diffusion measurement and interaction analysis of membrane molecules, and extends the ICS that can be analyzed by confocal fluorescence microscopy.

On the other hand, in mass spectrometry, we succeeded in separating not only detergent but also triglyceride from sample...
of glycolipids in adipocytes using the DCE washing method, and achieved structural analysis (32). If we use the adipose tissues derived diabetes patients and animal models, we will be able to verify our working hypothesis of lifestyle-related diseases, which is “the insulin receptor shifts from caveolae to GM3-rich domains in insulin-resistant adipocytes, thereby impair the insulin signaling.” (8) Furthermore, the DCE washing method is shown to be useful not only for the removal of nonionic detergents but also for the removal of the anionic detergents taurocholic acid and deoxycholic acid, and the amphoter ic ionic detergent, CHAPS (28). Therefore, this washing method is available and started to be used not only for the removal of the detergent after membrane microdomain fractionation, but also for other applications, including the recovery of glycolipids from glycosyltransferase, carbohydrate hydrolysis enzyme reactions, cell lysates, and removal of detergents from the reaction mix (33). In this article, analysis of glycolipid microdomains using fluorescence microscopy and mass spectrometry was described. In the future, we expect not only to improve existing instruments but also to introduce new technology, such as visualization of glycolipid microdomains at a spacial resolution exceeding a light diffraction limit using super-resolution microscope, and high resolution structure determination of biomolecules in solution using single particle cryo-electron microscopy. These will help to elucidate the principle of glycolipid microdomain construction completely.

References


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