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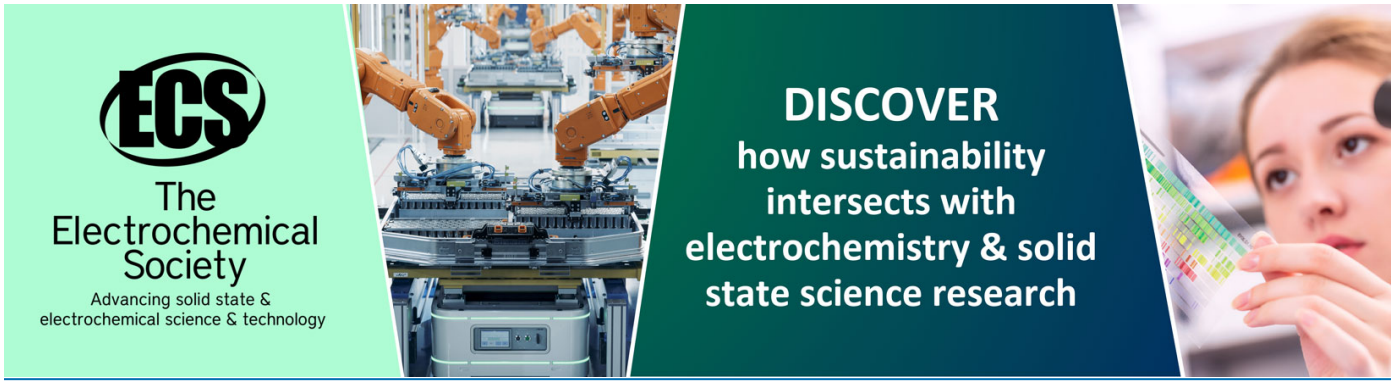

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Measuring the diffusion coefficient of ganglioside on cell membrane by fluorescence correlation spectroscopy

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Abstract The fluidity of proteins and lipids on cell membrane plays an important role in cell's physiological functions. Fluorescence correlation spectroscopy (FCS) is an effective technique to detect the rapid dynamic behaviors of proteins and/or lipids in living cells. In this study, we used the rhodamine6G solution to optimize the FCS system. And, cholera toxin B subunit (CT-B) was used to label ganglioside on living Hela cell membranes. The diffusion time and coefficients of ganglioside can be obtained through fitting the autocorrelation curve based on the model of two-dimensional cell membrane. The results showed that the diffusion coefficients of ganglioside distributed within a wide range. It revealed the lateral diffusion of lipids on cell membrane was inhomogeneous, which was due to different microstructures of cytoplasmic membrane. The study provides a helpful method for further studying the dynamic characteristics of proteins and lipids molecules on living cell membrane.

Keywords: diffusion coefficient, gangliosides, Hela cells, fluorescence correlation spectroscopy

1. Introduction

Lipid bilayers are vital self-assembled structure of cell to segregate different constituents and environments from the surrounding. It is essential to ensure life activities can be carried on in the cells. One of the essential properties is the fluidity. The fluidity of the lipids has great effects on the social life of cells, which include intracellular or extracellular signaling, transmembrane signal transduction, cell–cell or cell–extracellular matrix adhesion etc. [1]. Therefore, how does the fluidity of membrane regulate the physiological function of the cell has become the focus of the biology. However, the membrane lipids have various dynamic properties even in the same cell. The main reasons may come as follow: Due to the difference structure of lipid such as the head group, the length of the carbon chain, the degree of saturation of fatty acids, etc., there are thousands of known lipids in the membrane [2]. Because of entropy effects, the interaction between lipid tail and head group will lead the same lipids are more likely to act as similar dynamic characteristics. Another reason is the protein in the membrane can segregate specific groups of lipids. Hence, there is a difference in dynamic properties even the same lipid [3]. Finally, the diffusion of lipids will also be changed by the cytoskeleton and other structures in the process of cell migration, Endocytosis and exocytosis [4]. In summary, the dynamics of lipids are closely related to the physiological functions of membrane. Exploring the dynamic characteristics of lipids have gradually become an effective hotspot to understand the fluidity of lipid bilayers in the molecular level and reveal the physiological processes and regulatory mechanisms of the membrane.

The velocity of lipid molecules is too fast to capture in diffusion process. It requires that technique's temporal resolution is above millisecond. Fluorescence Recovery After Photobleaching (FRAR) is an important tool in previous studies to detect the fluidity of plasma membrane [5]. Fluorescence correlation spectroscopy (FCS) is also a versatile molecule technique [6]. We can obtain the diffusion coefficient of molecules, concentration, chemical reaction rates, and other parameters though autocorrelation analysis of the fluorescence intensity in the spot. Confocal-based FCS has become an effective mean to detect cell dynamics at the molecular level because of high temporal and spatial resolution and weak invasiveness.

Ganglioside is a kind of sphingolipid containing sialic acid. It exists in the mammalian cell membrane to protect cell membrane and promote the activity of enzyme. Ganglioside closely relate to membrane function which has attracted the attention of biologists. The diffusion coefficient of ganglioside was measured in this study. The parameters of facula were optimized by the rhodamine6G solution at the beginning of the experiment. And, cholera toxin B subunit (CT-B) was used to label the ganglioside on living HeLa cell. The

living cell was measured by FCS and the diffusion coefficient of ganglioside was obtained by autocorrelation calculation to achieve the dynamic characteristic of specific lipid.

2. Materials and methods

2.1 Materials

The system was TCS SP8 (Germany, Leica) equipped with a 63× water immersion objective (NA=1.2). It was composed by a confocal microscope, a couple of avalanche photo-detectors and correlators. The fluorophore was excited with a 488nm laser. The size of the FCS focus was limited by pinholes, which reduced the intensity of the background and improve the signal to noise ratio.

Two kinds of dyes were used for systematic calibration and lipid measurements on the membrane in this article. The Rhodamin6G was used to measure the volume of the spot in order to optimize the system. The aqueous solution is scarlet and glow a yellow-green fluorescence excited with a 488nm laser. The dyes for the measurement of lipid in the membrane are divided into two species, which are lipophilic dyes and specific marker dyes, respectively. The specific marker dye can attach to specific lipids in the membrane. However, the lipophilic dyes cannot reflect the diffusion coefficient of specific lipids and diffuse into certain areas. Cholera toxin from vibrio cholera is comprised of two subunits, A and B. The nontoxic B subunit is important to protein complex as it allows the protein to bind to cellular surfaces via the pentasaccharide chain of ganglioside G_{M1} to get the diffusion coefficient of ganglioside in living cell [7].

2.2 Methods

There are two ways to obtain the parameters of the confocal volume. It included the effective volume, lateral and longitudinal waist radius, which are related to the excitation wavelength, laser power, objective and imaging settings. One of the methods is calibrate the system with the known diffusion coefficient of solution. According the obtained diffusion time we can acquire the effective volume by FCS. The other is calibrated with a known concentration solution to obtain the number of molecules. We can calculate the size of the detected volume based on the concentration of sample. The former method was used in this study. Where the diffusion coefficient of Rhodamin6G solution was D = 2.8 *$10^{-6}$$cm^2$/ s at 22 ℃ [8].

We use auto-correlate curve to analyze the fluctuation of fluorescence intensity, so we need to select the appropriate fitting model for different samples. The normalized autocorrelation function (ACF) can be written as

$$G(t)=\frac{\langle \delta F(t)\delta F(t+\tau)\rangle}{\langle F(t)\rangle^2} \tag{1}$$

where the G(t) represent the correlation of fluctuations. <F(t)> is the average of fluorescence intensity. The simplest example of a diffusion process with a Gaussian propagator is simple Fickian diffusion, with a mean squared displacement linear in time,

$$MSD(t)=6Dt \tag{2}$$

where MSD(t) is mean squared displacement, the diffusion coefficient is given by the Stokes–Einstein relationship,

$$D=\frac{KT}{6\pi\eta R} \tag{3}$$

where R is the hydrodynamic radius of the particle, K is the fluid viscosity, ŋ is Boltzmann's constant and T is the absolute temperature. In this case, the normalized autocorrelation function obtained as

$$G(t)=\frac{1}{\bar{N}}(1+\frac{t}{\tau_D})^{-1}(1+\frac{t}{S^2\tau_D})^{-1/2} \tag{4}$$

the S is the ratio of the longitudinal waist to the lateral waist. The characteristic decay time associated with the diffusion of the fluorophore

$$\tau_D=\omega_0^2/(4D) \tag{5}$$

Since the triplet stage and the diffusion are not at the same time scale, we measure the data with a delay time about 0.008ms. We can obtain the arithmetic of volume though Eq (5) according to the time of lateral diffusion and the known diffusion coefficient of Rhodamin6G solution.

$$S=\frac{z_0}{w_0};V_{eff}=\pi^{3/2}w_0{}^2z_0;D_i=\frac{w_0{}^2}{4\tau_D}; \tag{6}$$

The two-dimensional detection volume can be calculated as

$$V_{2D}=\pi w_0{}^2/2 \tag{7}$$

The diffusion of lipid molecules in the membrane will deviate from the simplest Fickian diffusion under the influence of a variety of components and complex structures. A frequently discussed anomalous diffusion process with a Gaussian propagator is fractional Brownian motion [9]. A fractional Brownian motion process has a mean-squared displacement

$$MSD(t) = 6Dt^{\alpha} \tag{8}$$

The normalized autocorrelation function can be simplified as

$$G(t) = \frac{1}{\bar{N}}\left[1+\left(\frac{t}{\tau_D}\right)^{\alpha}\right]^{-1} \tag{9}$$

We have quantified the anomaly α and the residence time[10]

$$\tau_D = \left[\omega_0^2/(4D)\right]^{1/\alpha} \tag{10}$$

The diffusion coefficient also can be acquired. It has been shown to be a good approximation for a large class of anomalous diffusion processes when studied with single-scale FCS [10].

3. Results and Discussion

Before the experiment, the parameters of spot were measured by the rhodamine6G solution. We selected the different measurement points in the concentration of 2nM rhodamine6G solution and repeated the measurement several times. The autocorrelation curve was shown in Fig. 1. When the autocorrelation amplitude was attenuated to half, the diffusion time was obtained. The small residuals indicated that the model selection was appropriate. It is reliable to obtain the relevant parameters such as diffusion coefficient and diffusion time from the autocorrelation curve. The parameters were showed as table 1 according the Eq. (2) and Eq. (6). The results indicated the size of the focus was limited by pinholes. The system was good to measure the diffusion on the membrane.

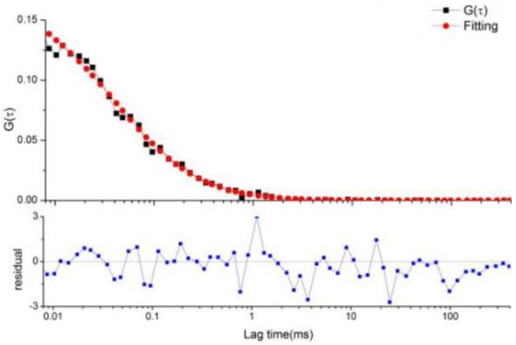

Fig.1. Autocorrelation curve of Rhodamin6G in solution.

Tab. 1. The data of referred volume V_{eff}

samples	τ_d (ms)	ω_0 (um)	S	Z_0 (um)	V_{eff} (fl)
1	0.023	0.225	4.051	0.911	0.091
2	0.018	0.201	3.591	0.719	0.057
3	0.035	0.28	3.832	1.073	0.165
4	0.036	0.285	4.546	1.296	0.207
5	0.052	0.341	3.248	1.109	0.254
6	0.041	0.303	3.775	1.145	0.207
Average	0.034 ±0.012	0.027 ±0.051	3.841 ±0.438	1.042 ±0.201	0.163 ±0.076

The Hela cells were stained for 30mins by cholera toxin subunit B conjugates. We measured the diffusion coefficient of lipid on the membrane in an optimized FCS system. The different autocorrelation curve showed different diffusion time in Fig. 2. The curves were fitted with extend model as Eq (9). Therefore we obtained the distribution of diffusion coefficients according hundreds of data (show in Fig.3). The specific lipid has a characteristic diffusion coefficient in model membrane. On the contrary, the results showed that the diffusion coefficients of ganglioside were inhomogeneous on the cell membrane. The diffusion coefficient obtained by lipid measurement in living cell was distributed in two orders of magnitude, indicating that there were two significant different kinds of diffusion in the membrane. There was a small amount (about 20%) of the data stay at a faster level, and the number of data greater than 0.6um^{2}s^{-1} was significantly reduced. It indicated not all of ganglioside diffused at slow level. On the contrary，the diffusion coefficients of ganglioside were aggregated in the range of 0.1-0.3um^{2}s^{-1}, which was similar to the protein in the membrane. The reason is the diffusion properties of specific marker dyes are usually associated with the type and location of labeled lipid. The B subunit of cholera toxin has proven to be a powerful tool for mark the lipid domains. The domain can associate with protein which usually segregate specific groups of lipids and form the platform to lead the cellular signaling. Hence, the slow diffusion kinetics may be associated with protein signaling in the membrane.

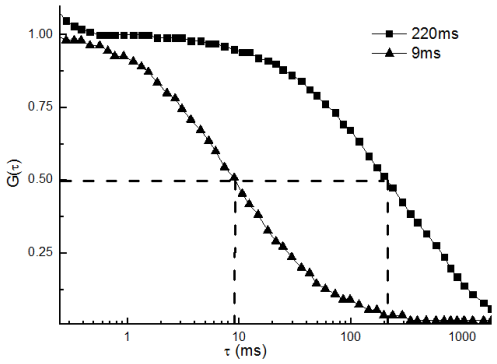

Fig.2. Autocorrelation curve of ganglioside on the membrane.

The results showed that fluorescence correlation spectroscopy can be used to quantitatively measure the diffusion coefficient of the cell membrane. Although membrane behavior is determined not only by the behavior of individual lipids but also the emergent properties of lipid collectives, a large number of measurements can also obtain the dynamic characteristic for the specific lipid. This study provided

an effective technical support for further study of the dynamic characteristics of proteins and lipid molecules in living cell membranes.

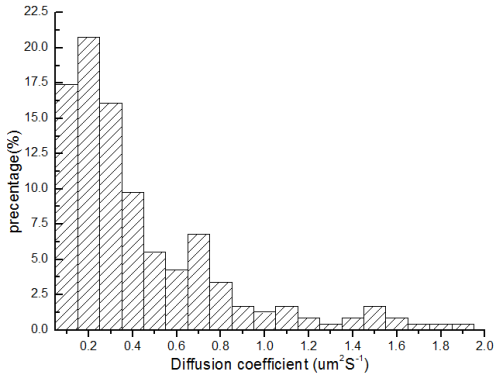

Fig.3. The distribution of diffusion coefficient.

4. Conclusions

We built up fluorescence correlation spectroscopy based on confocal microscope and discussed the feasibility of FCS to explore the molecular diffusion in the membrane. The results showed that the heterogeneity of the diffusion coefficient of ganglioside was obtained in a wide range. Then it was verified that there were various diffusion coefficients in the cell membrane even the same lipid. Different diffusion rates may correspond to the different physiological state of cell membranes. It also reflects the complexity and diversity of cell membrane structure. Above all, FCS is a promising technique to investigate the dynamic characteristic of membrane in living cell.

Acknowledgments

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