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Lipid−Protein Interactions Revealed by Two-Photon Microscopy and Fluorescence Correlation Spectroscopy

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ABSTRACT
Cellular processes involve a multitude of chemical reactions that must be kept in delicate equilibrium to maintain cellular homeostasis. Powerful biophysical techniques are needed to measure the localization and concentration of target molecules as well as to quantify complex molecular processes in model and *in vivo* systems. Two-photon microscopy and fluorescence correlation spectroscopy (FCS) can measure association and dynamics of appropriate molecules under equilibrium conditions. FCS provides information on motility (diffusion coefficients), concentration (number of particles), association (molecular brightness), and localization (image) of the target molecules. All of this information, in conjunction with computational modeling techniques, can help us to better understand the network of complex molecular interactions, which are at the basis of cellular processes. Fluorescence imaging techniques add the beauty of visualization to the scientific information. Photons emitted by a fluorescent dye are digitized, and the associated spatial information and intensity can be translated into different colors and shades providing the researcher not only with quantitative intensity information but also with spatial resolution and visual comprehension of two- or three-dimensional images. In this Account, we review the use of two-photon excitation microscopy and FCS in the study of lipid−protein interactions. We discuss these new methodologies and techniques, and we present examples of different complexity from qualitative to quantitative, from simple model systems to studies in living cells.

Introduction

Historical Overview. An historical overview of FCS, two-photon microscopy, and lipid−protein interactions takes us to the nineteenth century. At that time, two terms that we use in this Account were introduced: Brownian motion and cellular metabolism. In 1827, Scottish botanist Robert Brown experimentally demonstrated microscopic motion that was to become known as Brownian motion. Specifically, Brown suspended pollen grains in water and examined the solution closely. He noted that the water was "filled with particles" that were "very evidently in motion". This movement "arose neither from currents in the fluid, nor from its gradual evaporation, but belonged to the particle itself".[1] Later, in 1839, when the theory of spontaneous generation was still popular, two Germans, Matthias Schleiden and Theodor Schwann, made another observation: all living organisms are made up of cells, "elementary structures that, indeed, are subject to modification but are essentially identical−that is, they are cells". They recognized membranes, nuclei, and cell bodies to be common cell features. It was Theodor Schwann who coined the term "metabolism" to describe the chemical changes that take place in living tissue. A long time passed before techniques were developed that enabled scientists to relate Brownian motion to cellular metabolism. Some of these techniques are discussed in this Account.

Concerning the development of two-photon microscopy, the first theoretical studies in two-photon excitation were done by Maria Göppert-Mayer,[2] who submitted her doctoral dissertation in Göttingen on the theory of two-photon quantum transitions in atoms in 1931. However, the first experimental demonstration of the two-photon effect had to wait until 1961 for the invention of the laser, at which time two publications appeared simultaneously. Franjen and co-workers[3] showed that light from a ruby laser at 694 nm, which was incident into a quartz crystal, generated light at 347 nm. Kaiser and Garret[4] published the first report on two-photon excitation of $CaF_2:Eu^{2+}$.

About 10 years later, in 1972, fluorescence correlation spectroscopy (FCS) was introduced by Elson, Magde, and Webb.[5] In 1990, Denk and Webb[6] demonstrated a new type of microscope based on two-photon excitation of molecules. Finally, in 1995, Berland, So, and Gratton put together the two technologies, two-photon excitation microscopy and FCS, and demonstrated the potential of this methodology for intracellular measurements.[7]

Today, FCS is a widely used technique. On-line references (http://www.ncbi.nlm.nih.gov) using the FCS keyword gives more than 2700 hits. Approximately 2% of these papers correspond to FCS done with two-photon excitation, while the rest of these studies were done using one-photon excitation with normal confocal microscopes. Restricting the search to two-photon−FCS−lipid−protein interactions turns up around 20 articles published in the last 5 years.

Enrico Gratton was born in Italy and received his doctorate in physics from the University of Rome. Presently, Dr. Gratton is a professor in the Departments of Physics and Biophysics. In 1986, Dr. Gratton was awarded a grant from the National Institutes of Health to establish the first national facility dedicated to fluorescence spectroscopy: the Laboratory for Fluorescence Dynamics (LFD). The LFD, housed in Loomis Laboratory of Physics at UIUC, is a state-of-the-art fluorescence laboratory for use by local, national, and international scientists. Dr. Gratton's research interests include design of new fluorescence instruments, protein dynamics, hydration of proteins, and IR spectroscopy of biological substances. He has over 400 publications in referred scientific journals.

Susana Sánchez was born in Chile and received her doctorate degree in Chemistry from the Catholic University of Valparaiso. She came to the University of Illinois at Urbana-Champaign (UIUC) in 1997 as a postdoctoral fellow in the Laboratory for Fluorescence Dynamics (LFD) directed by Dr. Gratton. Since 2001, she has been the User Coordinator of the LFD. Dr. Sánchez is also a Research Scientist with research interests in the area of protein−protein and protein−lipid interactions. Her current project involves the use of two-photon microscopy for the study of cholesterol removal by HDL particles from membranes.

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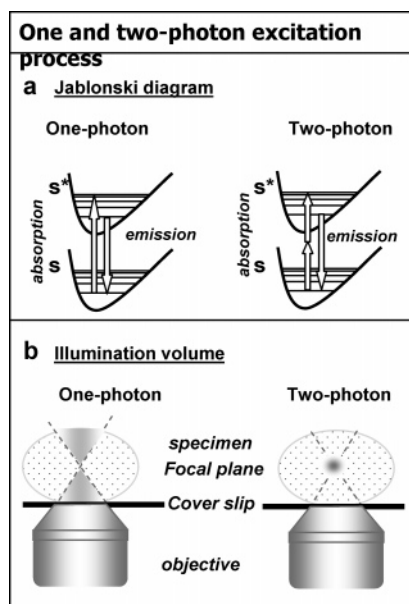
FIGURE 1. Excited state (S*) can be reached by absorption of one photon or two photons with half the energy of the energy gap (a). Because the emission wavelength is the same for one- and two-photon absorption, in the two-photon instruments, it is easier to separate excitation from emission. In the case of the two-photon excitation, the illumination volume is confined in the focal plane of the objective (b), providing an intrinsic optical sectioning capability without the use of pinholes.

Principles

Excellent articles have been published on the basis and mathematics of the two-photon excitation process[8−10] and on the FCS technique.[7,11−15] Here, we will refer to the basic principles involved in each technique, focusing on those parameters that will be important later in the discussion of the application of two-photon microscopy and FCS to lipid−protein interactions.

Two-Photon Excitation. Two-photon molecular excitation is a nonlinear process in which a chromophore simultaneously absorbs two incident photons. The energy of the photon pair should be equal to the energy required for excitation (Figure 1a). In the simplest case, each photon will provide half of the energy. The probability for this process to occur is very low. Two-photon cross sections are typically in the order of 10^{-50} cm^4 s photon^{-1}. If we compare the absorption rate for one- and two-photon processes, we can better appreciate how low this probability is. The absorption rate for one photon (k_1) is given by the one-photon absorption cross section (σ) and the single-photon excitation flux (φ_1) by

$$k_1 = \sigma \times \varphi_1 \tag{1}$$

The absorption rate for two photon (k_2) is given by the two-photon cross section (δ) and the square of the two-photon excitation flux (φ_2) by

$$k_2 = \delta \times [\varphi_2]^2 \tag{2}$$

For rhodamine, the cross sections for one- and two-photon excitation are 10^{-20} m^2 and 10^{-56} cm^4 s photon^{-1}, respectively, at 544 and 800 nm. If we consider a standard
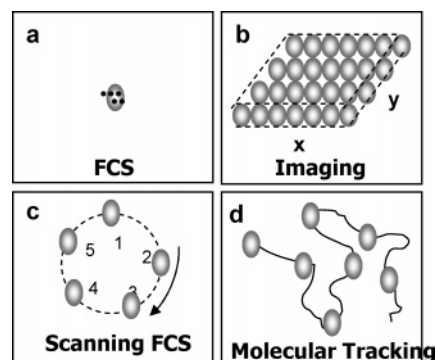
FIGURE 2. Two-photon illumination volume (shaded dot) can be moved in several modalities in the *x, y,* and *z* axes by using scanning mirrors, and this will define different two-photon techniques.

fluorescence measurement in a cuvette where the area of illumination is around 0.4×0.4 cm and a laser with an output power of 10 mW at the maximum absorption wavelength, eqs 1 and 2 predict that the absorption of one photon will occur every 6 ns, while the two-photon absorption will occur in 5000 years!

To increase the probability of the two-photon absorption, we can increase the density of excitation reaching the sample. The development of mode-locked, high-peak-power laser sources with femto- to picosecond pulses made it possible to have high instantaneous photon flux at the sample. However, this condition is not sufficient. If we illuminate a rhodamine solution with one of these lasers, we will not observe any appreciable two-photon excitation, rather we need to concentrate the photons even further using high numerical aperture (NA) objectives. The objective will concentrate the light, and the maximum density will be at the focal volume where enough intensity will be present for the two-photon excitation to occur (Figure 1b).

When the sample is illuminated with a pulsed, high repetition rate laser, the rate of two-photon excitation is expressed as the number of photons absorbed per fluorophore per pulse (η_a) and it is a function of the pulse duration (τ_p), the pulse repetition rate (f_p), the photon absorption cross section (δ), and the numerical aperture of the microscope objective (NA),[6] which is expressed as

$$\eta_a \approx \frac{P_0^{\,2}\delta}{\tau_p f_p^{\,2}}\left[\frac{\pi \mathrm{NA}^2}{hc\lambda}\right]^2 \tag{3}$$

The optical sectioning effect; i.e., only a small volume of the sample is excited, is inherent in a two-photon excitation microscope, and it originates during the excitation process because of the quadratic dependence of the excitation process on light intensity. Localized fluorescence excitation limits fluorophore bleaching and photodamage to the focal plane; therefore, the maximal observation time is prolonged. Another advantage of two-photon excitation for microscopy is that the wavelength of excitation and emission are far apart, resulting in low background noise.

Fluorescence Correlation Spectroscopy (FCS). The small excitation volume created by two-photon excitation

a

b

Autocorrelation function

c

τ(sec)
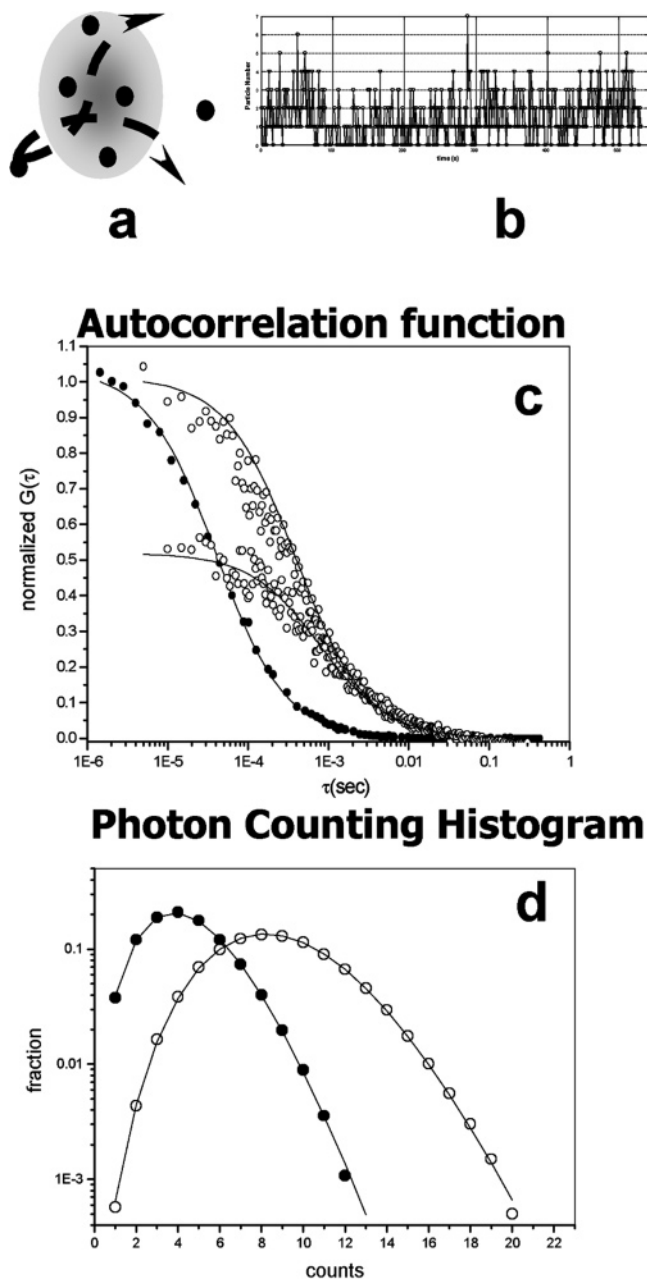
Photon Counting Histogram

d

counts

FIGURE 3. Diagram showing the principles of FCS acquisition and analysis. (a) The shaded big circle represents the nonhomogeneous illumination of the laser beam on the sample. The small black dots represent the target fluorescent particles going in and out of the illuminated volume according to their diffusion coefficient and mass. The fluctuations in fluorescence produced by the movement of the particles are register on time (b). (c) Autocorrelation curves correspond to fluoresceine (●) and Alexa488−tubuline (○) (d) PCH analysis for two concentrations of fluoresceine. Brightness per particle is the same, but the average number of particles is different: 4.44 (○) and 1.95 (●).

is key to several microscopic techniques (Figure 2). If the small illumination volume is kept immobile, one can observe molecules diffusing in and out of this volume (Figure 3a) and detect the fluorescence fluctuations as a function of time (Figure 3b). This technique is called FCS. The analysis of these fluctuations can be done by the autocorrelation function (ACF) or by the photon-counting histogram (PCH).

The ACF, $G(\tau)$, characterizes the time-dependent decay of the fluorescence fluctuations to their equilibrium value. In simple terms, ACF calculates the similarity between a signal $I(t)$ and a copy of the same signal shifted by a time lag τ, $I(t + \tau)$.

$$G(\tau) = \frac{\langle I(t) \times I(t + \tau) \rangle - \langle I(t) \rangle^2}{\langle I(t) \rangle^2} \quad (4)$$

From ACF, we can recover two parameters: the diffusion coefficient (D_{coef}) and the average number of particles in the observation volume ($\bar{N}$) given by the inverse of $G(0)$, multiplied by a constant that depends on the illumination profile. Figure 3c shows a diagram of three autocorrelation curves showing differences in $G(0)$ and diffusion coefficients. The two curves with a normalized $G(0)$ of 1 correspond to fluorescein and the tubulin dimer labeled with Alexa488 (the labeling pattern was such that each subunit had one fluorophore on average). These curves show clear differences in diffusion coefficients, namely, 300 and 54 $\mu m^2/s$ for fluorescein and Alexa488−tubulin, respectively.[16] The third curve, with a relative $G(0)$ of 0.5, corresponds to the same concentration of Alexa488−tubulin but in the presence of 1 M guanidinium chloride (Gdn-HCl). At this concentration of Gdn-HCl, the tubulin dimer is completely dissociated and hence the number of labeled particles doubles, which can be observed from the inverse of $G(0)$. Diffusion coefficients for proteins in solution depend on their molecular weight. In the case of monomer−dimer equilibrium, the difference between the diffusion coefficients of the two species is only 1.2 compared with the number of molecules in solution that changes by a factor of 2 when the equilibrium moves from all monomers to all dimers or vice-versa (and assuming that each subunit has one fluorescent label). Diffusion coefficient is a useful parameter to study molecular association, for example, when proteins bind to membranes. When proteins are in a membrane, the mobility is restricted and the values for D_{coef} decrease to $1-0.1$ $\mu m^2/s$ and the difference with the same protein in solution allows us to distinguish the two species.

For PCH analysis,[12,17] the probability of detecting photons per sampling time is calculated. The probability is experimentally determined by the histogram of the detected photons. In principle, the Poisson distribution describes the occupation number of particles that can freely go in and out a small excitation volume. However, the distribution of photon counts deviates from the Poisson distribution mainly because of the diffusion of molecules in the inhomogeneous excitation profile and the statistics of the photon-counting detector. The analysis of the distribution of photons is based on the deviation of the measured PCH from the expected Poisson distribution because of the molecule occupation number. Two parameters characterize the photon distribution: the number of molecules in the observation volume ($\bar{N}$) and the molecular brightness (β), which is defined as the average number of detected photons per molecule per second. Figure 3d shows the PCH analysis for two con-
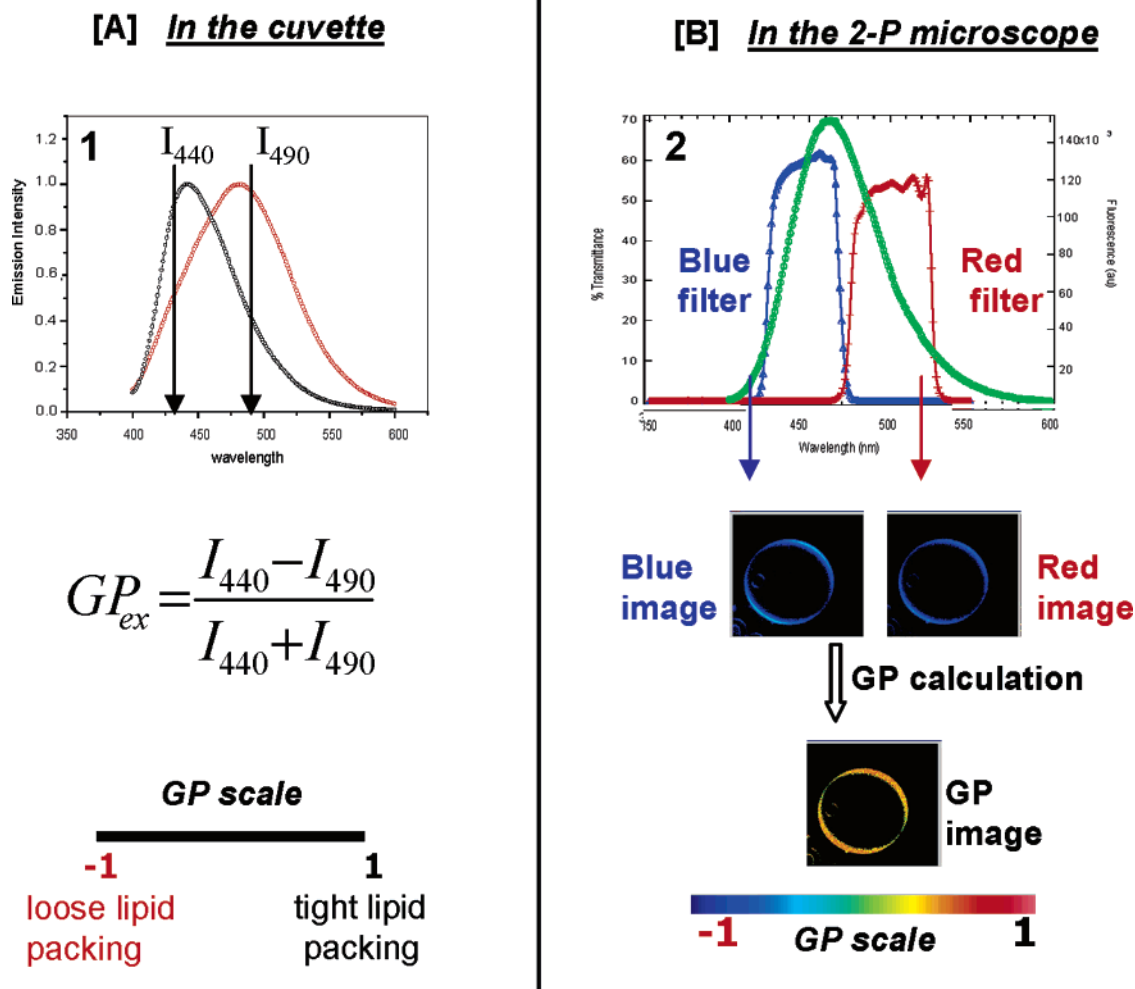
Laurdan GP measurements

[A] *In the cuvette*

$$GP_{ex} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

GP scale

-1 loose lipid packing   **1** tight lipid packing

[B] *In the 2-P microscope*

Blue filter   **Red filter**

Blue image   **Red image**

GP calculation

GP image

-1   **GP scale**   **1**

FIGURE 4. Laurdan emission in the phospholipid bilayer centers near 440 nm when the membrane is in the gel phase (A1, black line) and near 490 nm in the liquid crystalline phase (A1, red line). The GP function measures the emission shift. In a two-photon, dual-channel instrument, the excitation wavelength is typically 780 nm. The fluorescence is split into red and blue channels by a dichroic beam splitter and interference filters (B2). Separate detectors are used to simultaneously collect the blue and red emission. Red and blue images are then recombined to form the GP image of the sample in near real-time [SimFCS, Laboratory for Fluorescence Dynamics (LFD)].

centrations of fluorescein. Because the excitation power is constant, the brightness is the same but the particle number is different. PCH is extremely useful for analysis of fluctuations inside cells, where the target protein can associate in clusters or bind to other pre-existing aggregates.

FCS is based on the analysis of fluctuations of a few molecules in the excitation volume. Traditional confocal microscopy also provides a small excitation volume using pinholes. Therefore, FCS is a technique that can be carried out in solutions and in cells using traditional confocal microscopes, which are available in many laboratories. Because the focus of this Account is in two-photon excitation FCS, however, we will leave that area of the literature to the interested reader.

Imaging and Laurdan Generalized Polarization (GP). If the small excitation volume is moved in the x and y axes (Figure 2b), one obtains an optical slice with the width of the two-photon volume, in the z direction, normally around 1 μm. When the beam is moved in the z

direction, one creates a series of optical sections that can be reconstituted into a 3D image. Fluorescence images can also be obtained using different emission filters. In this way, we can spatially map two (or more) molecules, provided that they have been labeled with different dyes. A valuable use of the two-color imaging in two-photon microscopy is Laurdan GP imaging (Figure 4), used to study membrane fluidity. Laurdan [6-lauroyl-2-(dimethylamino) naphthalene] is used as a membrane probe because of its large excited-state dipole moment, which results in its ability to report the extent of water penetration into the bilayer. Water penetration has been correlated with lipid packing and membrane fluidity.[18,19] A full discussion on the use and mathematical significance of GP can be found in the literature.[19,20]

Other Techniques. If the beam is moved in a circular orbit (Figure 2c), one can collect FCS data at different points in the orbit to create a spatial map of diffusion coefficients and concentrations. If the measurements are done inside a cell, one can measure movements and

concentrations of molecules in the cytosol, in the membrane, inside particular organelle, etc. This technique has been named scanning FCS.[21] The laser beam can be moved to the position of maximum intensity to follow the particle in time (Figure 2d), tracking the particle center of mass with high precision.[22] These two techniques are just being developed in the LFD, and they promise to be very important for *in vivo* studies.

Lipid−Protein Interactions Using Two-Photon Microscopy and FCS

General Considerations. To study lipid−protein interactions by two-photon excitation microscopy, either the protein and/or the lipid must be labeled with a fluorophore. This labeling is not a trivial issue. First, a dye must be chosen that can be excited by two photons. The two-photon cross section of several dyes have been published.[14,23] The protocols for labeling proteins *in vitro* depend on the specific protein and the question to be answered. If the studies involve elucidating the oligomeric state of a protein, for example, it is necessary that all subunits are labeled and that the dyes do not change their fluorescence properties depending on the state of association.[16,24] Then, the number of molecules and the molecular brightness can be use to determine the association state.

When the studies are done using FCS, either in solution or *in vivo*, it is important to consider that the concentration used is much lower than that required in most other techniques. For instance, in normal fluorescence studies such as fluorescence intensity, emission wavelength shift, anisotropy, and lifetime, the experimental concentrations normally are in the range of 10^{-8} to 10^{-6} M. With a 1 μM solution in a standard 1 cm square fluorescence cuvette and if the light beam illuminates a volume of 160 μL (0.4 $\times$ 0.4 $\times$ 1 cm), we will excite about 9.6×10^{13} molecules. In FCS, the illumination volume is normally 1 fL (10^{-15} L). If we use the same micromolar concentration used for the cuvette experiments, we will be illuminating about 600 molecules. This number of molecules is too large to see fluctuations in intensity because of individual molecules entering or leaving the illumination volume. Normally, FCS works best with concentrations in the nanomolar range, i.e., from 1 to 6 molecules in the excitation volume. In practical terms, this condition means that FCS is most suitable to study equilibrium with dissociation constants in the nanomolar range or below.

Interactions with Artificial Membranes. To simplify studies involving membranes, artificial membrane systems are normally used. The basic idea is to construct a liposome that mimics in composition the natural membrane. In cuvette experiments, a commonly used lipid system is that of large unilamellar vesicles (LUVs). A more appropriate system for microscopic studies, because of their size, are the giant unilamellar vesicles (GUVs) with an average size in the range of $10-100$ μm that allows visualization of individual liposomes (Figure 5).

Most of the studies on lipid−protein interactions in the literature use LUVs as a membrane system, and if one
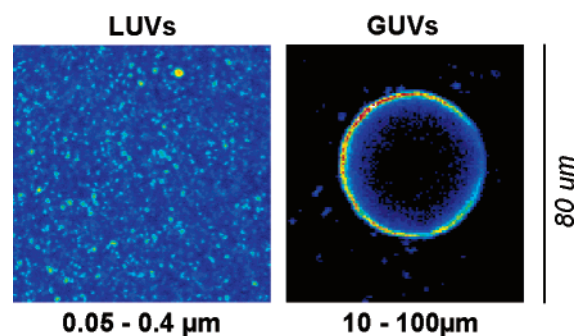
FIGURE 5. Comparison of membrane model systems. In the LUVs panel, the small dots are free-floating LUVs of average size smaller or comparable to the microscope resolution. The GUVs panel shows an optical section of a giant unilamellar vesicle.

wants to relate the LUVs and GUVs studies, it is important to consider two fundamental differences. First, the curvatures of these two types of liposomes differ, being smaller and more similar to cellular curvatures in the case of the GUVs. Second, when working with GUVs, one liposome at the time is observed; therefore, the phenomena being observed is probably due to individual behavior rather than the average behavior obtained when many LUVs are being observed simultaneously.

In our lab, we developed a method to grow GUVs[25] based on the electroformation method originally described by Angelova and Dimitrov in 1986.[26] One important demonstration, first obtained in our laboratory, is that GUVs can be constructed with pure lipids, mixtures of lipids, natural lipids extracted from cells, and also with whole membranes containing proteins and lipids.

Several types of studies can be done using the GUV model system (Figure 6). Once specific lipid domains in the GUV are recognized by the Laurdan GP, one can visualize protein binding to a specific domain just by inspecting the image. It is also possible to measure the diffusion of molecules in the different domains because they are large enough to be observed separately. Because the GUVs are clearly separated from the rest of the solution, the diffusion and concentration of the protein in the solvent can be measured. The availability of the GUV image makes it simple to ascertain if the interaction of the membrane with the protein produces changes in the morphology of the liposome such as shrinking, and the GP measurement can be quantified and localize changes in the packing of the lipids of the bilayer because of protein binding.

Interactions with Natural Membranes (Cells). For *in vivo* studies, autofluorescence plays a crucial role. The excitation wavelength and the dye used to label the target
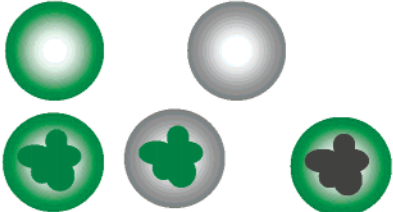
FIGURE 6. Schematic representation of experimental protocols used in the lipid−GUVs studies with a two-photon microscope. Circular objects represent the top view of the GUV with or without lipid domains. In the GP measurements, rings represent GUV cross sections and different colors represent regions of different water content.

molecule are selected considering the level of autofluorescence. A simple way to label the target protein is by molecular biology using the green fluorescent protein (GFP) technology. Genetic labeling guarantees that all synthesized proteins have one GFP. Overexpression of the molecules can be a problem because FCS requires only a few particles. Therefore, delicate control of the expression level is needed. The family of fluorescent proteins are the first choice for *in vivo* studies with confocal and two-photon microscopy.[27−30] Other strategies for *in vivo* labeling are permeation of labeled proteins, ballistic labeling, FLAsH (fluorescein arsenical helix binder) methodologies,[31] etc. Using the labeling strategies outlined above, cells are not uniformly labeled, and if the extent of labeling is low, finding the target cell can be challenging.

FCS measurements can be carried out in the cytosol, nucleus, or extracellular space. Particularly useful for cell work is the new technique of scanning FCS, where FCS measurements are performed almost simultaneously in

several parts of the cell, as described previously.[21] This method is discussed in more detail later.

Applications

Preparing the Arena. Two-photon excitation in microscopy studies of lipid protein interactions started in our lab in 1999, when Bagatolli and Gratton[25] combined two-photon excitation microscopy and the GUV vesicles methodology. They published a series of papers using this methodology together with different fluorescent dyes to study the properties of pure lipids and mixtures. For the first time, they showed images of the lipid phase separation in different lipid mixtures in unsupported bilayers, with lipid domains bigger than the pixel size.[32]

With the combination of two-photon microscopy and the GUV technology, the stage was set for the study of lipid−protein interactions (Figure 7). Two-photon microscopy and the GUVs protocol offered a system wherein binding could be observed directly, and the changes in
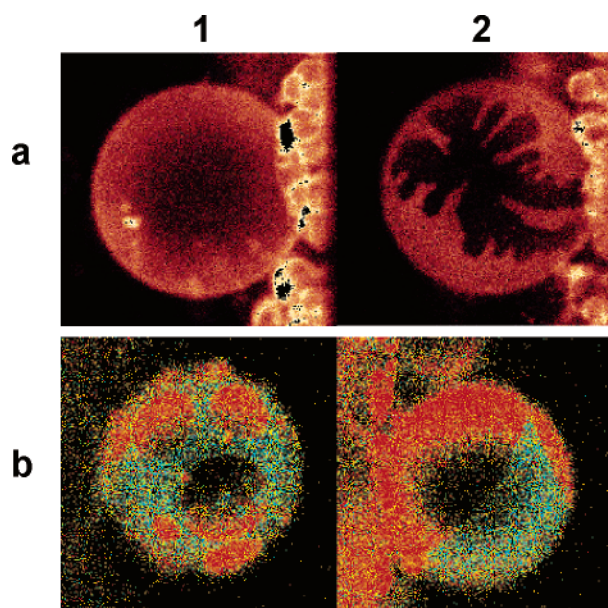
FIGURE 7. Scenario for studying lipid—protein interaction in two-photon microscopy in which the lipid phase is clearly distinguished. (a) Intensity images of Laurdan-labeled GUV made of (1) POPC/SPH 5:7 w/w over the transition temperature of the two lipids, at 38 °C, which does not show domain coexistence, and (2) the same mixture at 21 °C, which shows leaf-shaped black domains corresponding to more packed lipids. (b) GP images of (1) DOPC/DPPC 1:1 (mol/mol) plus 30% cholesterol at 18 °C and (2) the same mixture but at 14 °C. The images were taken at the LFD.

the membrane fluidity could be measured by Laurdan generalized polarization. Several proteins were used for these studies. In our lab, the phospholipase project, directed by Thoedore Hazlett, involved the study of the interactions of *Crotalus atrox* PLA$_2$ with pure and mixed lipid GUVs.[33] Two-photon excitation and Laurdan GP measurements were used to conclude that hydrolysis of POPC vesicles by the enzyme produced a dramatic shrinkage of the vesicles until they actually disappeared. Also, in a single GUV showing gel-liquid coexistence, the enzyme preferentially hydrolyzed the lipid in the liquid state.

Our laboratory established several collaborations with groups who wanted to study their systems using the two-photon microscopy techniques and the GUV methodology. Joseph Albanesi from UT Southwestern in Dallas, in collaboration with David Jameson from the University of Hawaii,[34] studied the binding of fluorescein-labeled dynamin II to POPC giant vesicles. A surprising finding was that dynamin II binds to vesicles in the absence of PI (4,5)-P2. Activation of the GTPase activity of dynamin II by pure POPC was then shown. Alejandra Tricerri and Ana Jonas[35] from the Biochemistry Department of UIUC complemented standard biochemistry studies such as chromatography and differential calorimetry with two-photon microscopy to show differential binding of apo A-I and two different sizes of HDL particles to POPC vesicles.

The complexity of the artificial mixtures used to make GUVs gradually increased as we learned to make GUVs with lipid extracted from cells and with complex lipid mixtures. Natural rafts (samples isolated by the Triton

X100 procedures) and rafts-like mixtures were studied alone and interacting with different proteins.[14] Sascha Janosch,[36] from the lab of Roland Winter in Germany, studied the lateral membrane organization and phase behavior of the lipid mixture DMPC/DSPC/cholesterol (0−33 mol %) with and without the incorporation of a lipidated peptide labeled with the fluorophore BODIPY and the lipid fluorophore N-Rh-DPPE [BODIPY-Gly-Cys-(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe]. In particular, this study shows the ability of the peptide to induce formation of fluid microdomains at physiologically relevant cholesterol concentrations.

Natural membranes were used in studies on erythrocytes done by John Bell, from Brigham Young University. Erythrocytes are susceptible to the action of sPLA$_2$ (secretory PLA$_2$), but the mechanism involved is unclear. It is known that elevation of intracellular Ca^{2+} is involved in the mechanism. Red blood cells pretreated with ionomycin were exposed to *Agkistrodon piscivorus piscivorus* sPLA$_2$, and the GP images show that ionomycin produces a clear heterogeneity in the cell membrane and that the existence of this heterogeneity is the key parameter for the susceptibility of the membranes to sPLA$_2$.[37,38]

The spatial resolution of two-photon microscopy and Laurdan GP measurements were used to show the existence of lipid rafts *in vivo*.[39] The existence of areas in the membranes with different fluidities, with sizes larger than a pixel, complemented experiments using specific raft markers in several cell types.

In the Way of Understanding Membrane Complexity. To study molecular interactions *in vivo*, the major challenge is understanding the complexity of the interaction network. Complex cell biological processes, such as cell cycle, apoptosis, signal transduction, intracellular trafficking, organelle biogenesis, and cell motility, are mediated by interactions among large numbers of different molecules. To understand these interactions, quantitative data on intracellular concentrations and diffusion constants for each molecular species are needed. FCS can provide this information, in particular, two-photon FCS.

FCS studies in artificial membranes paved the way for *in vivo* studies. We showed that in model systems the FCS illumination beam can be located in the liposome bilayer and provides information on the diffusion of proteins in the membrane. The two-photon excitation beam is larger than the membrane thickness. Therefore, if there are proteins in solution as well, the signal would be a mixture of the proteins in the two different compartments. If the difference in diffusion coefficients is large enough, the autocorrelation analysis will show two components in the analysis. In her Ph.D. thesis, LFD graduate student Qiao Qiao Ruan[30] studied the cellular location of adenylate kinase and its isoforms, genetically labeled with EGFP (enhanced GFP) and expressed in CHO cells. FCS measurements were done to determine the mobility (diffusion coefficient) and the concentration (particle number) in solution and in the membrane.

Particularly useful in cellular studies is the two-channel FCS cross-correlation technique, where molecules are

labeled with dyes that emit in different spectral regions and the emission signal is divided by a dichroic mirror and appropriated filters. In this way, fluctuation from the two channels can be separately measured. LFD researchers, along with researchers from McGill University and Royal Victoria Hospital in Canada,[40] worked with fluorescence resonance energy transfer and FCS in CHO-K1 cells and somatostatin receptors. They demonstrated the presence of monomeric, homooligomeric, and heterooligomeric receptor species in the same cell cotransfected with the two receptors.

The FCS work described so far was done focusing the laser beam in one point at a time. With our newer instrumentation, we can take FCS data almost simultaneously from several points in the cell using the scanning FCS technique. Scanning FCS was first introduced in our lab, and already two publications have resulted. One publication describes the methodology as applied to studies of GUVs and CHO cells,[21] and the other describes an application to the determination of partitioning of NaPi cotransporters and the modulation of its diffusion by membrane composition.[41]

Final Remarks

In this Account, we describe the two-photon microscopy methods used to study membrane−protein interactions. We show that using the GUVs model one can detect binding of proteins to different membrane phases by simple inspection of images. The different phases can be distinguished by the GP value of the Laurdan probe or by using different probes that partition differentially between the two phases. The FCS methodology adds the quantitative evaluation of the number of molecules in a given excitation volume, either free in solution or bound to the membrane, thereby providing partition coefficients. Furthermore, because of the different diffusion coefficient of proteins in solution and in the membrane, FCS can unequivocally establish if a protein is free in solution or associated with the membrane. Two-photon microscopy and FCS are particularly suitable for *in vivo* studies. The scanning FCS methodology further increases the number of locations where the FCS experiments can be performed at the same time and further reduces photobleaching, because the laser beam is moved to a different location after a very brief period of time. Two-photon excitation and the various FCS methodologies are impacting the way in which protein−membrane interactions are studied, both *in vitro* and *in vivo*.

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