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Multiphoton Fluorescence Microscopy

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Multiphoton fluorescence microscopy has now become a relatively common tool among biophysicists and biologists. The intrinsic sectioning achievable by multiphoton excitation provides a simple means to excite a small volume inside cells and tissues. Multiphoton microscopes have a simplified optical path in the emission side due to the lack of an emission pinhole, which is necessary with normal confocal microscopes. This article illustrates examples in which this advantage in the simplified optics is exploited to achieve a new type of measurements. First, dual-emission wavelength measurements are used to identify regions of different phase domains in giant vesicles and to perform fluctuation experiments at specific locations in the membrane. Second, we show how dual-wavelength measurements are used in conjunction with scanning fluctuation analysis to measure the changes in the geometry of the domains and the incipient formation of gel domains when the temperature of the giant vesicles is gradually lowered. © 2001 Academic Press

Key Words: multiphoton excitation; two-photon microscopy; fluctuation correlation spectroscopy; giant unilamellar vesicles; scanning fluctuation correlation spectroscopy; generalized polarization; Laurdan.

Several reviews have been published recently about multiphoton microscopy by our group and by others (1–8). This article is intended to be a guide to practical applications of multiphoton microscopy. In particular we discuss the possibilities offered by this technique for quantitative image acquisition and single- or multiple-point fluctuation analysis at specific pixels of the image. We first describe some important features of the instrument setup, which enables this mode of operation. We present, using examples taken from experiments performed in our laboratory, some of the unique features of multiphoton microscopy. In addition we discuss common methodological problems encountered during the course of these measurements.

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In the context of single-point measurements in conjunction with image analysis, the most striking difference between a conventional confocal system and a multiphoton microscope is that in the latter case the emitted light does not need to travel through the confocal pinhole to reach the detector. This different optical configuration makes it possible, with relatively simple optics, to use several colors in the emission arm without being affected by color aberrations of the objective. This alone is a distinct advantage. We also note that multiphoton excitation is particularly good at exciting several common fluorophores using a single excitation wavelength. In addition, the collection of the emitted light with a minimum of optical components in the emission path increases light collection efficiency. We performed experiments to compare the light losses under typical conditions due to the combined effect of optical path/aberrations and we concluded that such losses could be quite substantial in a standard confocal instrument compared with the multiphoton system. One additional advantage of our multiphoton system compared with the confocal is the capability to stop the beam at any point during the raster scan and to perform single-point measurements on a small volume of the sample. Of course, this option is not unique to multiphoton systems, but very few manufacturers of confocal microscopes offer this possibility, mostly because of instabilities in the beam steering mechanism.

TYPICAL MULTIPHOTON MICROSCOPY SETUP

To acquaint the reader with the basic building blocks of our multiphoton microscope, a schematic of our system is shown in Fig. 1. A unique feature of our microscope system is that the light detector can operate either in photon mode or in analog mode with a high-precision 12-bit analog-to-digital converter depending

on the choice of the operator. Another unique feature is that we can perform a range of different types of quantitative measurements just by using different parts of the software program but with no changes in the hardware or optical configuration (Fig. 1). Our microscope uses a titanium:sapphire laser as the source for two-photon excitation. The laser is tunable from 710 to 990 nm with a single mirror set. The fluorescence is measured directly without a pinhole in the emission arm. The system, built around the Zeiss Axiovert M100 microscope, has a Cambridge Technology galvano-scanner and high-sensitivity photomultipliers (Hamamatsu R928) that can operate both in the analog mode for lifetime measurements and in the photon counting

mode when high sensitivity is needed. With this microscope it is possible to acquire images as well as fluctuation data and to program the scanner to perform various types of sub- and full-frame scanning. The possibility of simultaneously acquiring images and fluctuation data is unique to our multiphoton microscope setup.

TWO-PHOTON CROSS SECTION OF SOME COMMON PROBES

First we show two-photon cross-section measurements we have performed in our laboratory using the microscope described on a series of common dyes used in fluorescence microscopy. This collection of two-photon spectra (Fig. 2) is similar to other measurements performed by other researchers, for example, the methods and results of Xu *et al.* (9–11), and illustrates the range of compounds that can be excited by a common excitation wavelength. The experimental system we used for this determination is the same experimental setup illustrated in the previous section, but the titanium:sapphire laser was operated in the picosecond mode to minimize errors due to wavelength-dependent pulse duration due to the dispersion in the optical elements of the microscope. The pulses had a bandwidth of approximately 1 nm and the duration ranged between 1 and 2 ps depending on the wavelength.

We sinusoidally modulated the laser intensity at 100 Hz using an electrooptic modulator. The laser wavelength was measured using a wavemeter (IST Rees)

FIG. 1. Schematic diagram of the two-photon microscope.

FIG. 2. Two-photon excitation cross section (product of two-photon fluorescence quantum efficiency and two-photon absorption cross section).

and the pulse duration with an autocorrelator (Femtochrome). The excitation light is split and sent to the sample and to a reference detector. In this way, we can measure the excitation intensity and extract the quadratic dependence of the fluorescence as a function of the excitation power while we scan the laser wavelength. Sample concentrations were determined using manufacturers' or published extinction coefficients.

As previously noted also by others, a single excitation wavelength in the region 720–800 nm could be used to excite common UV probes, such as Laurdan (6-lauroyl-2-dimethylaminonaphthalene), and ANS, as well as the visible probes fluorescein and rhodamine. This feature seems to be unique to the two-photon excitation process. In contrast, the fluorescence emission of all the compounds shown in the figure is the same as that obtained using one-photon excitation. Also the fluorescence lifetime is the same. However, fluorescence anisotropy can be different with two-photon excitation compared with one-photon excitation. For some compounds such as Laurdan, fluorescein, and rhodamine, the value of the initial anisotropy is larger for two-photon excitation than for one-photon excitation.

DUAL-COLOR SINGLE-POINT MEASUREMENTS AND IMAGES

We now discuss features and advantages of multiphoton microscopy by way of some examples of the different measurement modes that the microscope offers. In this

section we report some illustrative examples of dual-color measurements. The first example is from the field of membrane studies. It involves the use of the fluorescent probe Laurdan to detect and recognize domain regions of different phases in a giant unilamellar vesicle composed of two phospholipids that show phase immiscibility in a temperature region close to and below the main phase transition of one of the two phospholipids. The second example, from the field of scanning fluctuation correlation spectroscopy, is issued to show measurements of rapid spatiotemporal fluctuations in the same artificial membrane system obtained with the giant unilamellar vesicle method.

LIPID DOMAINS IN GIANT UNILAMELLAR VESICLES

To illustrate the quantitation that can be obtained using dual emission we report some recent measurements of lipid phase coexistence in giant unilamellar vesicles composed of an equimolar preparation of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DPPC/DLPC) (1:1, mol/mol). The phase diagram of this mixture was previously reported (12, 13). At temperatures above the gel–liquid crystal transition of DPPC (42°C), a single homogeneous phase is reported for this mixture, while at temperatures below the phase transition of DPPC, two separate phases have been reported. Our first aim is to determine if, in this regime, the regions of the

FIG. 3. Giant unilamellar vesicle composed of 1:1 mole ratio of DLPC and DPPC at 37°C. Left: Intensity image obtained at the top of the vesicle using a bandpass filter at 490 nm. Right: Generalized polarization (GP) image of the same vesicle. The regions inside the squares labeled 1 and 2 were used to calculate the histograms of GP values in Fig. 4. The image size is about $40 \times 40 \mu\text{m}$. The GP color scale spans from -1 to $+1$. Excitation was at 780 nm.

vesicles that can be identified as being in the liquid crystalline phase, and that are supposed to contain essentially DLPC molecules, have the same properties as vesicles that contain only DLPC.

Laurdan is a membrane probe that shows equal partition in the liquid crystal and gel phases of phosphatidylcholine phospholipids. The emission spectrum maximum of Laurdan changes from about 440 nm in the gel phase to 490 nm in the liquid crystalline phase. This change is due to water penetration in the membrane causing a different membrane packing in the liquid-crystalline phase compared with the gel phase. However, the shift toward the red of the emission spectrum of Laurdan in the liquid crystalline phase is a dynamic process. After excitation, the emission spectrum starts to move toward the red due to the changes in orientation of the water molecules in the immediate surroundings of the excited Laurdan molecule. This process depends on several factors, including temperature and amount of water in the membrane. As a consequence, the average amount of spectral shift during the excited state lifetime is exquisitely sensitive to the dynamics and water content of the membrane.

Figure 3 shows a two-photon (and hence sectioned) image of a giant unilamellar vesicle. The z section is obtained at the top of the vesicle. This image was obtained at 36°C, where relatively large, stable gel domains are formed. The left-hand part of Fig. 3 is the fluorescence intensity measured using a bandpass filter in the detection path centered at 490 nm. We also have another light collection channel with a bandpass filter centered at 440 nm. The dark regions in the left-hand panel of Fig. 3 correspond to gel domains. In these ordered regions, Laurdan molecules are aligned with their dipole transition almost exactly along the membrane normal (in the more central part of the frame).

Due to the direction of light propagation, which is along the z direction, no excitation can occur. The bright regions in the left-hand panel of Fig. 3 correspond to disordered liquid-crystalline regions. In this case, Laurdan molecules are only partially oriented along the membrane normal. The membrane disorder produces a population of Laurdan molecules with a relatively large component of the transition dipole moment parallel to the membrane normal and then excitation can occur. Although the intensity figure seems to show a relatively homogeneous liquid-crystalline phase, for the purpose of the illustration of the dual-wavelength method, we analyze the distribution of "spectral shifts" using the generalized polarization (GP) function. We can then

compare this spectral shift with the spectral shift measured in a pure DLPC sample and assess the heterogeneity of the lipid phase on the length scale of the point spread function by analyzing the GP pixel histogram.

ANALYSIS OF THE GP HISTOGRAM

The GP function was originally introduced by Parassassi *et al.* in 1990 (12) and used in conjunction with membrane studies using spectral-dependent lipophilic probes such as Laurdan and Prodan. The GP function is defined as

$$GP = \frac{I_B - I_R}{I_B + I_R},$$

where I_B is the intensity measured in the blue part of the spectrum, typically at 440 nm for Laurdan, and I_R is the intensity measured in the red part of the spectrum, typically at 490 nm for Laurdan. The GP function is mathematically related to the more common ratio-metric measurement. However, in the field of microscopy the additive propriety of the GP function (14) is an advantage. In contrast to cuvette measurements, where there is only one value of the GP at any given temperature, in an image of a vesicle it is possible to distinguish regions of different GP values.

The right-hand part of Fig. 3 corresponds to the GP image. Note that in our instrument we display the GP image as we acquire the image. The color scale maps the GP between 1 and -1 . Typically, pure gel phase has a GP around 0.5–0.6, while in the liquid crystalline phase the GP value is between -0.3 and -0.4 , depending on the temperature and water content of the membrane. The right-hand part of the figure clearly shows that the GP value is not homogeneous. A typical histogram of GP pixel values obtained in a region corresponding to the liquid crystalline phase (Fig. 4; region

FIG. 4. Histogram analysis of the GP function in two different regions (indicated by the regions labeled 1 and 2 in Fig. 3) of the giant vesicle. Left: Region 1; Right: Region 2.

indicated with square 1 in Fig. 3) gives an average value of -0.42 , with a relatively broad distribution. This average GP value is what should be expected from a liquid-crystalline phase according to cuvette measurements. Instead, other regions of the image, indicated with square 2 on the right-hand part of the figure, exhibit GP values in the range 0.26 , also with a relatively broad distribution. This value is definitively lower than the 0.5 – 0.6 value expected for a gel phase according to cuvette measurements. The GP image analysis clearly distinguishes regions of the membrane that have very different GP values and unequivocally identifies which regions are in the gel and in the liquid crystalline phase. The observation that the border of the field of view in the direction at 20 – 30° from the vertical in the image tends to have larger values of the GP is due to a combination of photoselection and polarization effects since in this region of the image we start to excite Laurdan molecules that are normal to the membrane surface because of the curvature of the vesicle.

The distribution of GP values in the different pixels of the image implies that on the membrane there is a distribution of gel phase, liquid-crystalline phase, and regions of intermediate order. It has been previously reported that the GP distribution in the gel phase is relatively narrow, while it is quite broad in the liquid crystalline phase. We confirm this observation and we add that in mixed phases obtained with 1:1 DLPC: DPPC, the gel segregated phase also has a relatively broad GP distribution.

FLUCTUATION CORRELATION SPECTROSCOPY (FCS) AT SELECTED POINTS IN AN IMAGE

In our multiphoton microscope it is possible to perform a fluctuation correlation measurement at a specific point of the image. For this illustration, we chose a point in the liquid-crystalline phase at about the center of square 1 in Fig. 3. We recorded synchronously for several minutes the intensity fluctuations in both detection channels. The intensity autocorrelation spectrum of one channel is shown in Fig. 5. The characteristic time for the decay of the intensity fluctuations is about 0.1 s. The fluctuations in both channels are correlated, indicating that the intensity fluctuations are not due to lipid phase fluctuations. Indeed, the autocorrelation function of the GP (blue line) shows a flat spectrum. However, other experiments (not shown here) show that there are regions in the image (close to the borders between the gel and the liquid crystal) where we can measure a definite autocorrelation spectrum of the GP

fluctuations that indicate phase fluctuations of the membrane, as shown in the next section.

SCANNING FCS AND THE FORMATION/ MOVEMENT OF LIPID DOMAINS

A unique feature of our multiphoton microscope is the capability to perform rapid scans of a certain part of an image. Scanning FCS in conjunction with multiphoton microscopy was first realized by Berland *et al.* (15). For this application, we performed a scan along a circular orbit. The period of the scan for each orbit was 2 ms. By properly aligning the points in time and space, we can have a quasi-simultaneous record of the changes at different spatial locations. If we consider the same spatial location, we can perform fluctuation analysis with a time resolution of 2 ms. The most important feature is that we can spatially correlate measurements at different locations.

Figure 6 illustrates a typical situation in which the image taken at the top section of a giant vesicle shows the existence of gel domains characterized by the absence of excitation (dark regions) and by the larger value of the GP as shown in the right-hand panel of Fig. 6. This image was obtained at 39.5°C , during the cooling cycle, when separated gel domains just start to form. The circle in the figure shows approximately the scanning path. To present the scanning data, we constructed a pseudo-image in which the x axis represents points along the orbit and the y axis represents the time.

Figure 7 should not be interpreted as an image, since the vertical axis represents the time evolution. However, using this pseudo-image, we can easily distinguish

FIG. 5. Autocorrelation function of the intensity fluctuations (black) and of the GP function (blue).

the spatial correlations and the evolution of the domains. Of course, if it were possible to acquire images at a very high rate, we would have even more information. However, as far as we know, two-photon images with a time resolution of 2 ms have not been achieved yet and the technique we propose provides most of the information without modifying or adding new hardware to the multiphoton microscope. What is interesting in this experiment is the possibility of measuring relatively fast fluctuations and spatially correlating the fluctuations at different points in an image. The same experiment could be performed on a cell and the fluorescence changes could be due to pH or calcium changes. In the experiment shown in Fig. 7 we can detect the onset of the formation of gel domains and follow their evolution in space and time. Although for this particular experiment the dynamics are relatively slow (on the second time scale) data were acquired on the millisecond time scale. The minimum rotation period for the scanner used in our multiphoton instrument is about 0.2 ms, allowing in principle the measurement of submillisecond dynamics.

DISCUSSION

The examples we have presented show typical applications of multiphoton microscopy to different biophysical and biological problems. The power of the method is due mainly to the elimination of the confocal emission pinhole typical of standard laser confocal microscopes. This simplification of the optical design has brought

together into a conventional fluorescence microscope a number of techniques that were used only in specialized instrumentation before, for example, imaging and fluctuation spectroscopy. A number of other methodologies, also possible in standard confocal microscopy, are basically simplified, for example, dual-emission analysis and lifetime imaging. One obvious question is whether or not one should get a multiphoton microscope. In this review we have discussed only multiphoton microscopy using dual-emission detection and for fluctuation analysis. Multiphoton microscopy also has advantages for a single-emission measurement, which has been previously discussed (4, 15–32), in terms of the simplified optical design, photobleaching characteristics, and quality of image formation. The limits of spatial resolution that can be achieved with multiphoton microscopy, also in conjunction with the 4-Pi setup, have been discussed (33–38). The most appropriate laser sources have also been discussed and schemes using both pulsed and continuous-wave lasers have been described (39–43). As the complexity of biological experiments increases and the number of questions a biologist asks during a microscopy experiment increases, simplification of the optical design seems to be a crucial requirement to make the system capable of multiple measurement modalities (44–53). For example, in the only commercial system available today (Confocor 2 from Zeiss) that combines confocal imaging with fluctuation analysis using one-photon excitation, the optical design is very complex. To change from the imaging mode to the FCS mode, the optical path must be changed in the Confocor 2. Instead, in our multiphoton system, the

FIG. 6. Left: Intensity image obtained at the top of a giant vesicle composed of 1:1 mole concentration of DLPC and DPPC. The image size is approximately 40 μm . The black circle in the figure shows the orbit of the circular scanning. Right: GP image corresponding to the left image. The GP color scale is from -1 to $+1$.

optical system is the same in both modes of operation. The only changes are in the way the data stream is treated by the software. In cases in which the biological experiment requires performing FCS measurements at selected locations in an image (in 3D space) the multiphoton system provides distinctive advantages. These advantages are augmented if dual-emission measurements are also needed. Dual- or multiple-emission measurements produce substantial benefits for the detection, interpretation, and quantitation of image features.

In the example we presented of GP measurements in giant unilamellar vesicles, the same measurements could have been made using a standard confocal microscope. The increase in sensitivity achieved in the two-photon microscope because of the simplified emission optical path is crucial to this kind of measurement. Furthermore, in the standard confocal microscope, color aberrations and color misalignments due to the dual-excitation and dual-emission characteristics of this kind of measurement can produce severe artifacts. These artifacts need to be recognized and properly treated. On the contrary, the multiphoton system, which generally uses a single excitation wavelength, is relatively free of artifacts. Calibration of filter transmission and detector relative sensitivity using solutions is acceptable.

In our opinion, however, one of the major advantages of the multiphoton system is the flexibility in choosing the measurement modality brought in by the simplification of the optical design. What other users and we value the most in our multiphoton system is that the microscope offers a number of measurement options without changing any hardware. The examples we have shown illustrate this point.

FIG. 7. Representation of the scanning FCS data. The x axis corresponds to points along the circular orbit and the y axis corresponds to different time points. For this image, 100 orbits were averaged per each line in the y direction. The total duration of the experiment was about 40 s.

CONCLUSION

The differences between standard one-photon confocal microscopy and multiphoton microscopy are discussed in the context of multiemission measurements and fluctuation correlation measurements in cells. The simplified optical design of the multiphoton system which lacks the emission pinhole and has a different emission path permits the use of several measurement modalities in the same setup and during the course of a single experiment. This is a unique capability, which, combined with other advantages of multiphoton excitation, allows a new class of experiments to be performed on cells.

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