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# MONTE CARLO SIMULATION OF MULTIFOCAL STOCHASTIC SCANNING SYSTEM

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Multifocal multiphoton microscopy (MMM) has greatly improved the utilization of excitation light and imaging speed due to parallel multiphoton excitation of the samples and simultaneous detection of the signals, which allows it to perform three-dimensional fast fluorescence imaging. Stochastic scanning can provide continuous, uniform and high-speed excitation of the sample, which makes it a suitable scanning scheme for MMM. In this paper, the graphical programming language — LabVIEW is used to achieve stochastic scanning of the two-dimensional galvo scanners by using white noise signals to control the $x$ and $y$ mirrors independently. Moreover, the stochastic scanning process is simulated by using Monte Carlo method. Our results show that MMM can avoid oversampling or subsampling in the scanning area and meet the requirements of uniform sampling by stochastically scanning the individual units of the $N \times N$ foci array. Therefore, continuous and uniform scanning in the whole field of view is implemented.

*Keywords*: Multifocal multiphoton microscopy; stochastic scanning; galvo scanners; Monte Carlo method.

## 1. Introduction

Fluorescence microscopy has become an important tool in the field of biomedicine.[1,2] Multifocal multiphoton microscopy (MMM) which was developed in the late 20th century, can improve the utilization ratio of excitation light energy and achieve video-rate imaging speed at high spatial resolution through the use of microlens array,[3–6] cascaded beam splitters[7] or diffractive optical element (DOE)[8] to produce a multifocal array on the sample. MMM employs parallel processing ability of optical system to excite the sample and detect fluorescence emission from multiple foci at the same time. MMM also has almost all the advantages of multiphoton microscopy, such as inherent optical sectioning and less photobleaching and photodamage to the out-of-focus sample. In recent years, it has attracted more and more research interests.

In the conventional MMM system, a pair of galvo scanners are generally used to realize two-dimensional (2D) beam scanning. Unfortunately, the speed of galvo scanners is slow and their scanning accuracy is not high enough in the case of rapid scanning. When they are used in the MMM system, oversampling or subsampling on the boundaries of each small region may occur and artifacts in brickwork pattern may be yielded, which will compromise the quantitative analysis of the research object. In order to obtain high-speed 3D imaging and study dynamic particle tracking, Jureller *et al.* developed a stochastic scanning MMM (SS-MMM) fluorescence imaging technique,[9] in which a $10 \times 10$ hexagonal focus array was produced using a DOE and then scanned with white noise driven galvanometers. With the SS-MMM system, uniform, rapid and continuous scanning of the sample was obtained. In recent years, other scanning methods and their applications in microscopy have also emerged. Zeng developed a random access two-photon fluorescence microscope based on acousto-optic deflector, which allows for the scanning of femtosecond laser on the area of interest of the sample with high speed and high precision.[10,11] Shao *et al.* developed an MMM system based on a spatial light modulator,[12,13] which has random access to any arbitrary regions of interest in the field of view (FOV).

In this paper, we propose a SS-MMM system. We use LabVIEW to achieve stochastic scanning of the 2D galvo scanners by using white noise signals to control the $x$ and $y$ mirrors independently. Moreover, the stochastic scanning process is simulated by using the Monte Carlo method. By optimizing the scanning control parameters, the problem of oversampling and subsampling on the boundaries of each small region is solved, the edge sampling artifacts are avoided, uniform, continuous and rapid stochastic scanning is obtained and the scanning efficiency is improved.

## 2. Experimental Setup

Figure 1 shows the schematic of the SS-MMM system. The titanium: sapphire laser provides mode-locked, ultra-fast femtosecond pulses with tunable wavelength from 700 nm to 980 nm. The output laser beam is expanded by the beam expander (BE) and then illuminates a DOE to produce $N \times N$ diffraction-limited foci array on the focal plane of lens L1. A pair of galvo scanners (GX and GY) are used to scan the $N \times N$ spots in the focal plane of the objective. The microscope objective then produces a pattern of independent high-resolution foci on the sample for simultaneous multiphoton excitation. The fluorescence emitted from the foci array is collected by the objective and separated from the scattered laser light with a dichroic mirror (DM) and a near infrared (NIR) blocking filter (BF). Then the two-photon fluorescence image of the sample is acquired by a charge-coupled device (CCD) camera.

In order to improve the scanning uniformity and continuity and also increase scanning speed, the galvo scanners (GX, GY) are driven by analog signals

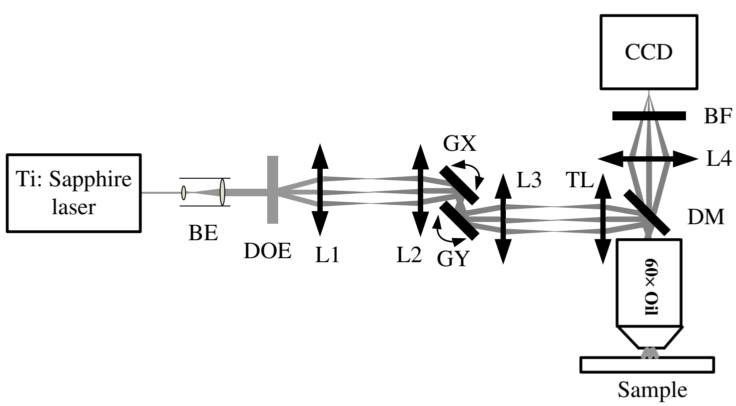



Fig. 1. Schematic of stochastic scanning multifocal multiphoton microscopic system; DOE: diffraction optical element; BE: expander; DM: dichroic mirror; TL: tube lens; GX, GY: galvo mirrors; L1–L4: lenses; BF: block filter.

produced by white noise signal generator and perform stochastic scanning on the sample. According to the specific experimental conditions (such as the distance between the foci), by measuring and analyzing the power spectrum of white noise, and with the computer simulation of the stochastic scanning process, we can optimize the scanning control parameters and reasonably select voltage amplitude and scanning time. Thus, continuous and uniform scanning in the whole FOV is implemented.

## 3. Stochastic Scanning Control System

A pair of galvo scanners are used in the system to achieve 2D beam scanning. The disadvantages of the galvanometer scanner are its slow speed and low accuracy in rapid scanning. When galvanometer is used in MMM system, oversampling and subsampling on the boundary of each small region and the resulting brickwork patterns in the image should be avoided. If a computer is employed to control the galvanometer for scanning Lissajous pattern on a sample, uniform, continuous and rapid scanning may be realized by independently changing the amplitude and the frequency of the scanning signal. If the $x$- and $y$- galvanometers are controlled independently by the analog white noise produced by signal generator, uniform and continuous "stochastic scanning" can be obtained.

However, stochastic scanning is affected by the size of the scanning area, distribution pattern of the foci and the optimization of the galvanometer parameters such as amplitude. When the GX and GY scanning galvanometers are controlled independently by analog white noise, from a statistical point of view, in the scanning area of each focus the dwelling time of the excitation light at each pixel obeys Gaussian distribution. The longer the scanning time, and the shorter the focus distance, the more possible we can get continuous and uniform illumination. Therefore, by decreasing the focus distance, the standard deviation of the corresponding residence time distribution of the stochastic scanning pixels in each scanning area is also reduced. Moreover, the scanning speed is significantly improved at the same excitation luminance.

We develop a LabVIEW program for stochastic scanning of the 2D galvanometers. Figure 2 shows the interface of scanning control module. The upper part is the diagram of the analog white noise driving signal that controls the GX and GY scanning galvanometers independently. The lower left part is

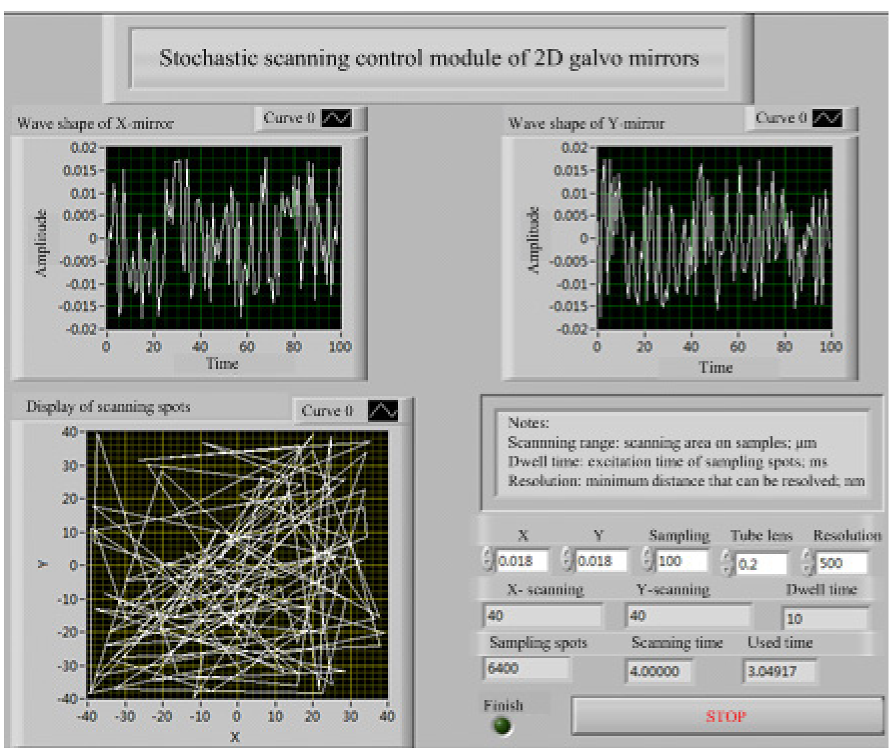



Fig. 2. Interface of scanning control module for 2D galvanometer scanners.

the distribution of the stochastic scanning point in the FOV. In addition, users can input many parameters such as voltage amplitude of the driving signal of the GX and GY galvanometer, the sampling number, resolution, etc. according to the experimental requirements. When the program is running, horizontal and vertical scanning scope, sampling number, single-point-dwell time and total scanning time will be displayed.

## 4. Monte Carlo Numerical Simulation of the Stochastic Scanning Process

Monte Carlo method, or the computer stochastic simulation method, is a calculation method based on "stochastic number". It can be simply understood as that when a large number of stochastic experiments are carried out, the frequency of the random events can be used as the probability.[14,15] Based on the Monte Carlo numerical simulation of the 2D galvanometer for stochastic scanning, we can realize continuous, uniform and rapid scanning of the sample. In order to achieve a large number of trials, algorithms mentioned in this paper have been performed for a large amount of cycles. Here we simulate the scanning process of spot array.

Firstly, we analyze the case of point-to-point scanning. The scanning area is assumed as a $3 \times 3$ square array with foci distance of $30\,\mu$m. In the process of point-to-point scanning, the step length on $x$- and $y$- direction is $1\,\mu$m, which means that each direction should be scanned 90 times and in the whole square area, 8100 points will be scanned. As to sequential scanning, we must measure or calculate the distance between the points precisely; while the difference between the dwelling time on the end-term points and the intermediate points can result in oversampling or subsampling and even brickwork pattern in the scanning image. As to stochastic scanning, we can only scan the area of interest and the dwelling time of the exciting light for every point is equal. If a uniform and continuous scanning image of the whole FOV is required, the scanned points should be more than 8100 points for the sequential scanning. Also, the scanning area should be generally larger than the FOV.

The diagrammatic sketch of $3 \times 3$ array scanning region is shown in Fig. 3. We name the small area for each point as a unit FOV. The following results are obtained by simulation using Matlab7. The

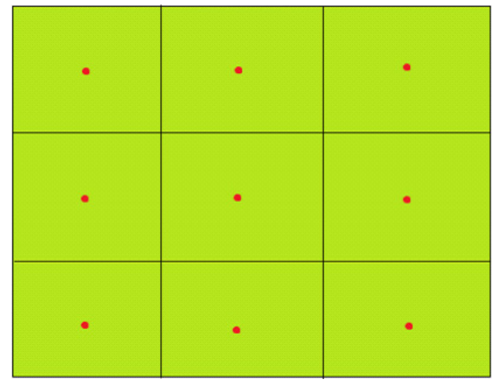


Fig. 3. Diagrammatic sketch of $3 \times 3$ array scanning region.

center of the scanning area is defined as the coordinate origin (0, 0). Based on this point, the coordinate of generated stochastic number is $(x, y)$. According to the distance between the points and the setting way for the sampling points, the coordinates for the other 8 points are determined at the same time, i.e.,

$$\begin{pmatrix} (x-30, y+30) & (x, y+30) & (x+30, y+30) \\ (x-30, y) & (x, y) & (x+30, y) \\ (x-30, y-30) & (x, y-30) & (x+30, y-30) \end{pmatrix}.$$

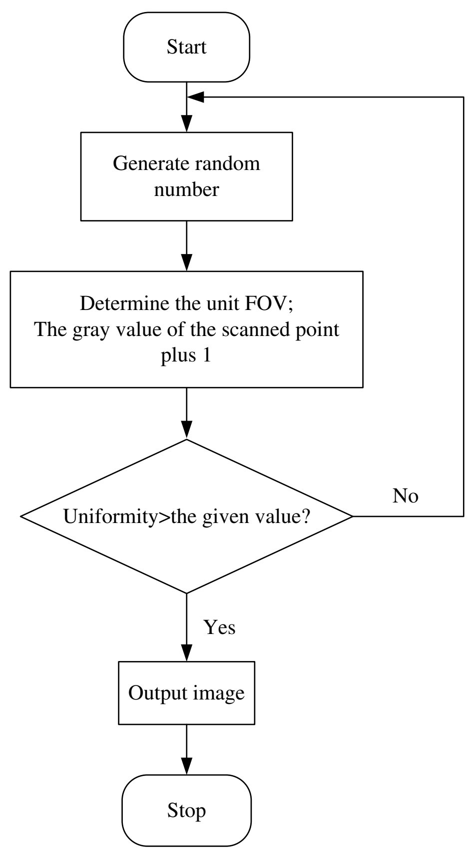



Fig. 4. Flow chart of stochastic scanning program.

Each time the stochastic number is generated in the central unit, the other 8 points will change at the same time. So the gray value of 9 points will be added 1 every time until the gray value satisfies the requirement of uniformity in each small area. Finally, we will get a grayscale image. Flow chart of stochastic scanning program is shown in Fig. 4.

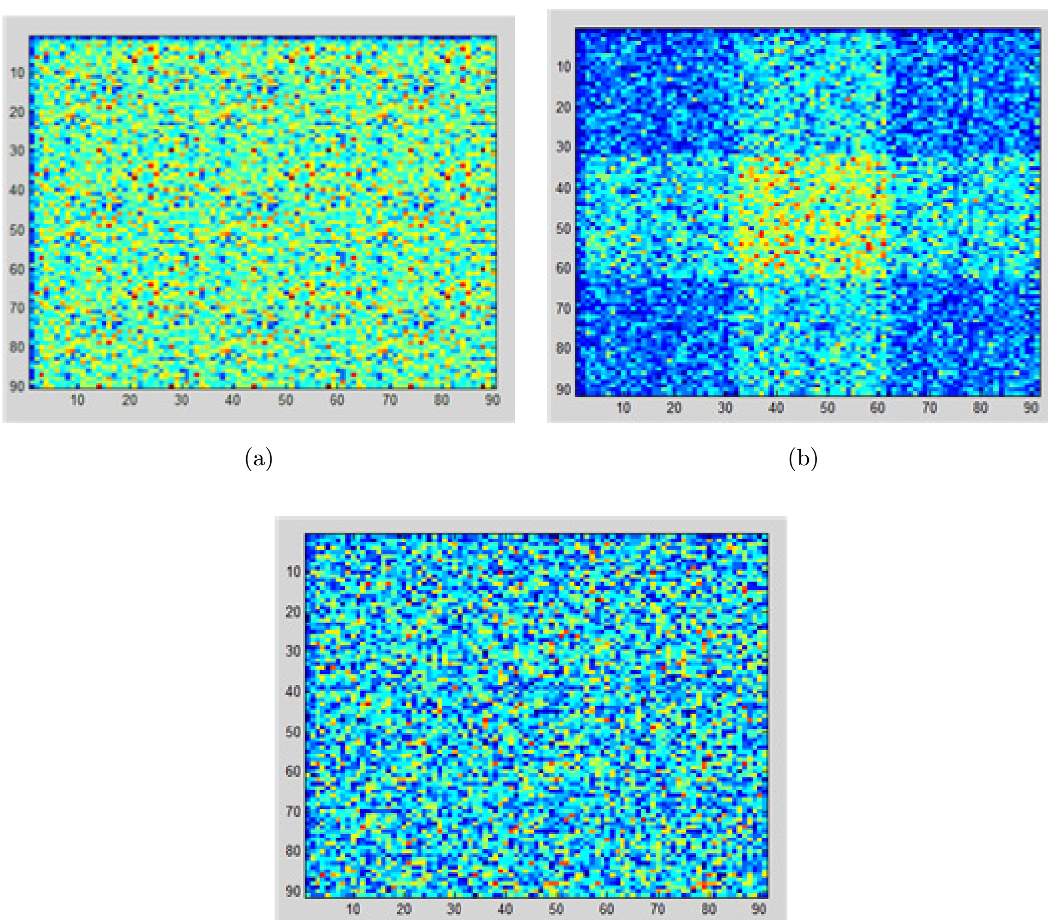
First, we set the range of stochastic number as $[-15, 15]$ for the central point, namely the values of $x$ and $y$ are in the range of $[-15, 15]$. The value range of the other points is the same so that the stochastic points will be limited within their units. The program is run and the grayscale image is shown in Fig. 5(a). The image shows slight "brickwork pattern" at the small grid border, but the unevenness of the overall image is less than 2%.

Then, we set the range of stochastic number as $[-45,45]$, which means that the scanning area will be expanded by 33%. The result is shown in Fig. 5(b). From the image we can see obvious grid distribution (or brickwork pattern) in the whole FOV.

At the end, the range of the stochastic number is further extended to $[-75, 75]$, which means that the scanning area is expanded by 67% compared to that in $[-15, 15]$. The result is shown in Fig. 5(c). In this case, the mean value of the unevenness over 10-time simulation is 1.4275 according to the following equation

$$\xi = \sqrt{\frac{\sum (I_i - \bar{I})^2}{n}} \Big/ \bar{I},$$

where $\xi$ is the unevenness of the whole FOV, $I_i$ is the intensity of the $i$th spot $(i = 1, 2, \ldots, n)$ and $\bar{I}$ is the average intensity of all the spots. The value we obtained is much better than the unevenness mentioned in the literatures.[3,4] From these images we



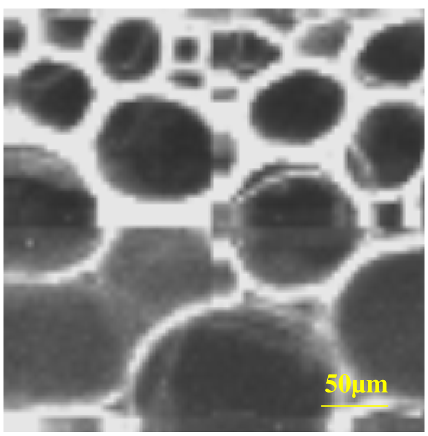

(a) (b) (c)

Fig. 5. Simulation results with stochastic number ranges of (a) $[-15, 15]$, (b) $[-45, 45]$ and (c) $[-75, 75]$, respectively.

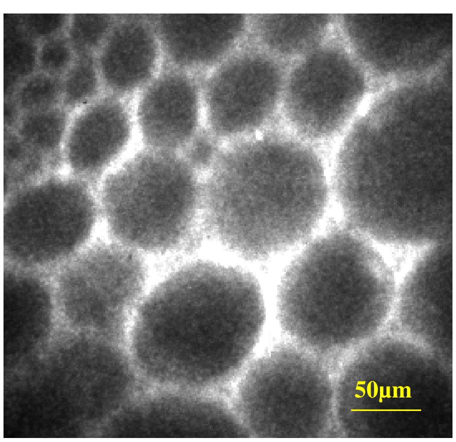
can see that the uneven phenomenon appearing in the above two situations is improved greatly and the sampling in the whole FOV is uniform and continuous. This result shows that when the scanning area is expanded by 67%, we can achieve continuous and uniform sampling.

But in practical experiments, when considering the imaging speed, we perform stochastic scanning within their respective units. And the simulation results show that the unevenness of the image in the stochastic scanning mode is much lower than that in the sequential scanning mode, which can meet the requirement of MMM for imaging uniformity. In Fig. 6, we give the two-photon excitation fluorescence images of prepared plant stem slide obtained by bi-directional raster scanning mode and stochastic scanning mode, respectively. The images are reconstructed from $2 \times 2$ array data sets and the total spots are about 4300. In our experiments, we set same acquisition time and same scanning spots for the two images. From Fig. 6(a), the obvious grid distribution and some oversampling phenomenon can be seen, while there is no noticeable mismatch in Fig. 6(b) by using stochastic scanning. Therefore, stochastic scanning has higher scanning efficiency over sequential scanning and there is almost no extra post-processing workload for stochastic scanning mode.

When the scanning area is fixed, increasing the points of the scanning array can greatly improve the imaging speed.




(a)




(b)

Fig. 6. Two-photon fluorescence images of prepared plant stem slide obtained by (a) bi-directional raster scanning mode and (b) stochastic scanning mode, respectively. The scale bar is 50 $\mu$m.

## 5. Conclusion

We develop a SS-MMM system that combines a DOE and a pair of galvo scanners for scanning. LabVIEW programming is used to achieve stochastic scanning of 2D galvo scanners and implement a friendly graphic user interface (GUI) for system parameter adjustments. The stochastic scanning is achieved by using white noise signals to control the $x$ and $y$ mirrors independently. Moreover, the stochastic scanning process is simulated using the Monte Carlo method using Matlab. We analyze the distribution of the image produced in different stochastic scanning areas in the case of $3 \times 3$ array. The results show that better, continuous and uniform illumination can be realized when the scanning area is expended by 67%. While in practical experiments, SS-MMM can meet the requirements of uniform sampling by scanning the individual unit of the $N \times N$ foci array, which can improve the imaging speed greatly. We compare the two reconstructed fluorescence images that are obtained by bi-directional raster scanning mode and stochastic scanning mode, respectively, and we find no noticeable mismatch in the image based on the latter mode. In this point of view, by using stochastic scanning, the scanning efficiency is improved and the post-processing workload can be reduced.

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