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Sub-diffraction imaging with confocal fluorescence microscopy by stochastic photobleaching

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ABSTRACT

We propose a single molecule localization method which takes advantage of stochastic photobleaching to improve the resolution of confocal fluorescence microscopy. By detecting the stochastic intensity loss of fluorophores, each fluorophore in the field can be localized. When all locations are known, a sub-diffraction image can be retrieved through single molecule localization algorithms. A confocal scheme is used to record the bleaching process of the sample. Each fluorophore can be localized from the recorded streaming followed by image subtraction. Compared with other single molecule localization concepts such as stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM), this method does not require a laser cycling equipment and the pixel size is no longer limited by the size of CCD. This technique works well with common fluorescent dyes and does not require the use of engineered photoactivatable proteins or photoswitchable synthetic dye pairs.

1. Introduction

Far field optical microscopy has long been a very important tool in life science because of its specificity, non-invasiveness and high imaging contrast. To eliminate the influence of the out-of-focus light, confocal microscopy was first put forward in the 1960s [1]. A pinhole was put in the sample's conjugated focal plane to exclude much of the out-of-focus light. However, resolution of conventional optical microscopy is constrained to the diffraction limit according to Abbe's theory [2].

In principle, fluorescence microscopy is capable of achieving unlimited resolution and many superresolution concepts related have already been proposed. Techniques based on engineering the effective point spread function (PSF) have received great attentions these years, which mainly include stimulated emission depletion microscopy (STED) [3–5], saturated structured-illumination microscopy (SSIM) [6,7], ground state depletion microscopy (GSD) [8,9], etc. STED, SSIM and GSD are based on nonlinear optical effects to improve their resolution. Despite the fact that great improvements in imaging resolution have been achieved, complex systems and expensive modules in these systems prevent these methods from being used widely. On the other hand, single molecule localization techniques achieve an impressive spatial resolution with simpler systems [10].

The family of single molecule localization microscopy techniques mainly involves photoactivated localization microscopy (PALM) [11–13], stochastic optical reconstruction microscopy (STORM) [14,15], etc. PALM and STORM overcome the diffraction limit by using stochastic activation of photoactivatable or photoswitchable probes, respectively. Each molecule can be localized precisely and a super-resolution image can be achieved by combining all the locations of the molecules. However, both PALM and STORM need to be equipped with a laser cycling system and special fluorescent dyes. When acquiring images with a fluorescence microscope, the overall fluorescence intensity decays because of the fluorescence photobleaching. However, photobleaching is also a stochastic process that can be taken advantage of. By detecting the stochastic intensity loss of fluorophores, every fluorophore in the field can be localized in theory. When all localizations are known, a sub-diffraction image can be retrieved by single molecule localization algorithms. Related superresolution concepts taking advantage of stochastic photobleaching have been proposed in recent years [16–20]. A technique named SHRImP (single-molecule high-resolution imaging with photobleaching) was first reported to localize two fluorophores within diffraction-limited spot images in 2004 [16]. In the same year, a similar method called NALMS (nanometer-localized multiple single-molecule microscopy) was reported, by which up to five molecules in a diffraction spot could be individually detected [17]. However, neither SHRImP nor NALMS could handle molecules at a high density. To map out the distribution of single molecules at high density, gSHRImP (generalized SHRImP) and BaLM (bleaching/blinking assisted localization microscopy) were

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put forward in the same year of 2011, and a resolution of 30 nm and 50 nm have been achieved respectively [18,19]. A similar method called PiMP (photobleaching microscopy with non-linear processing) was published in the next year. PiMP allows multi-color, three-dimensional sub-diffraction imaging of cells using common fluorophores [20]. However, except for PiMP most of the concepts mentioned above are based on a wide field scheme, where a total internal reflection fluorescence (TIRF) module and a high quantum efficiency CCD (EMCCD, for example) are needed. Thus, the pixel size is limited by the size of CCD, which prevents localization errors from being further reduced.

Here, we present a single molecule localization method based on the confocal scheme and the stochastic photobleaching. The experimental setup is simple. After recording the bleaching process of the sample by a confocal fluorescence microscope, single molecules can be localized from the recorded streaming followed by image subtraction. Compared with single molecule localization methods mentioned above, this method does not require a laser cycling equipment, works well with common fluorescent dyes and does not require the use of engineered photoactivatable proteins or photoswitchable synthetic dye pairs. The pixel size used is no longer limited by the size of CCD and analysis algorithms used for PALM or STORM can be transplanted to this method. Despite the fact that its higher background noise makes it hard to keep up with PALM and STORM in resolution for now, the simple concept and easy operation still make this method a competitive choice.

2. Set up

Schematic of the experimental setup is shown in Fig. 1. The excitation beam is generated by a continuous wave (CW) laser (Multi Channel Fiber Coupled Laser Source, Thorlabs) at a wavelength of 488 nm and is coupled into a single mode fiber (SMF) before expanded and collimated by the collimator (C). After passing through the dichroic mirror (DM), the excitation beam is injected into a galvanometric scanning system (GM) (Thorlabs). The dichroic mirror (DM) is used to separate the excitation beam and the fluorescence. The galvanometric scanning system (GM) is used to accomplish the scanning on the sample in two dimensions. After passing through the galvanometer system, the excitation beam is aligned by a lens and then focused on the sample by an objective lens (Olympus, 100 ×, 1.4 NA, oil immersion) (OL). The sample is fixed on a stage. The fluorescence emitted by the sample is collected by the same objective lens (OL). Until reflected by the dichroic mirror (DM), the fluorescence propagates along the opposite direction in which the excitation beam goes. The reflected fluorescence is collected by a lens-fiber system. A pinhole (P) is put in the sample's conjugated focal plane to exclude much of the out-of-focus light. The filter (FF03-525/50-25 from Semrock) used in front of the photomultiplier tube (PMT) (Thorlabs) is to make sure that only photons at the desired wavelength can be transmitted and detected. An imaging software (ThorImageLS v1.0) housed in the PC is used to retrieve the image from the intensity information provided by the PMT and the position information provided by the GM.

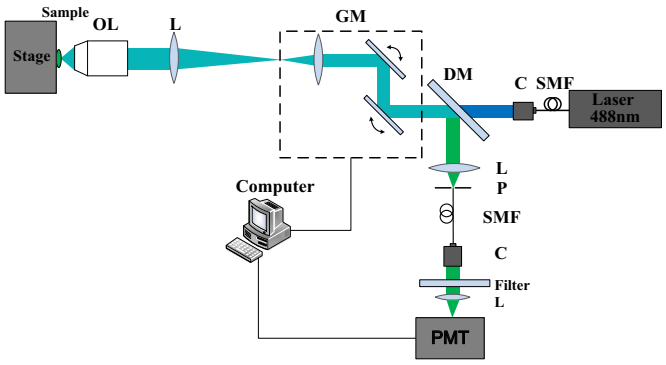

Fig. 1. Schematic of the experimental setup: SMF (single mode fiber), C (collimator), DM (dichroic mirror), GM (galvanometric scanning system), L (lens), OL (objective lens), P (pinhole), and PMT (photomultiplier tube).

Scanning parameters should be set under the following principles:

2.1. Pixel size

Different from PALM and STORM, this method suffers from higher noise caused by the subtraction. To distinguish signals from the noise, small pixel sizes should be used in case of pixel saturation [18]. Smaller pixel sizes makes it more accurate to localize fluorophores. According to [18], a pixel size of no more than 60 nm is recommended. Compared with a wide field fluorescence microscopy in which the pixel size is limited by the size of CCD, the confocal scanning scheme enables a wider range of pixel sizes.

2.2. Excitation power and scanning speed

To make sure that only a sparse distribution of fluorophores are photobleached between two successive frames, the excitation power should be set low and the scanning speed should be fast enough. On the other hand, to resist the effect of sample drifting, a relatively higher excitation beam power and a faster scanning speed are recommended to shorten the recording time. Thus, when the scanning area is given, one should choose the fastest scanning speed available in the system (512*512, 30.1 fps in our system), and the excitation power should be set in accordance with the scanning speed to make sure that only a sparse distribution of fluorophores are photobleached between two successive frames.

2.3. Gain of PMT

This method requires both a good signal strength and a fast scanning speed. A good signal strength can be achieved by increasing the dwell time or increasing the gain of the PMT (the bias voltage of the PMT). However, increasing the dwell-time may slow down the scanning speed. As a result, a PMT gain is often used. The gain value may differ from different samples. One has to adjust the gain value according to the scanning speed and the excitation power to make sure that the initial signal reaches the highest value permitted (the signal should not be saturated). In our system, the image format is uint16 TIFF, which means the initial signal should not be more than 65536 counts.

3. Data analysis

The general workflow for data analysis is shown in Fig. 2. First, a confocal scheme shown in Fig. 1 is used to record the whole process of fluorophores' photobleaching (till all the fluorophores are photobleached). For a confocal scheme, fluorophores' PSFs within the diffraction limit blend with each other and cannot be distinguished. Second, the sparsely-distributed image sequence is obtained by repeatedly subtracting the n-th frame from the $(n-1)$-th frame. Because of the stochastic nature of photobleaching, the visible population of fluorophores decays during the recording. The “missing” fluorophores' locations are also stochastic (even within the diffraction limit). By subtracting the current frame with its former one, one can obtain a sparse distribution