Background suppression by axially selective activation in single-molecule localization microscopy

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Resolution of a few tens of nanometers has been achieved in fluorescence microscopy with photoswitchable molecules. However, for thick samples, the background brought by the crosstalk of unwanted on-state molecules is nonnegligible. Now we present a background suppression method by using two axial standing waves generated by the interference of two activation beams with the same phases and two deactivation beams with the opposite phases. With spatially incoherent illumination, most activated molecules are located in a thin layer. The performance of such method is simulated with the known photoswitching characteristic of Cy5. With suitable parameters, the thickness of the layer can reach 39 nm (FWHM). © 2010 Optical Society of America

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Diffraction-limited resolution in optical microscopy has been surpassed in a group of single-molecule localization microscopy (SMLM) methods, such as photoactivated localization microscopy (PALM) [1], stochastic optical reconstruction microscopy (STORM) [2], fluorescence photoactivation localization microscopy (fPALM) [3], PALM with independently running acquisition (PALMIRA) [4], and direct STORM (dSTORM) [5]. They share a similar concept that the final super-resolution image is generated by superimposing many resulting images at different times, and in each resulting image, a sparse subset of fluorescent molecules are activated and imaged according to their precise locations by centroid localization.

However, spatial resolution in the third dimension does not benefit from single-molecule localization directly. Thus axial localization methods [6–9] are usually combined with SMLM to extend the high resolution to the third dimension. Although several inspiring images have been acquired by using these techniques, three-dimensional (3D) nanoimaging of thick samples, such as a whole cell, remains a challenge. The reason is obvious that, as a method relying on detection and localization of single molecules, background brought by the crosstalk will decrease the localization accuracy dramatically. To suppress such background, very thin samples, or background suppression techniques such as total internal reflection fluorescence, should be used. However, for thick samples, background is still a tough problem. There are two ways to minimize the influence of background on resolution. One is selective imaging. Only a layer of activated fluorescent molecules that are fully separate with each other are kept in imaging process, while those molecules activated but in crosstalk are discarded. It is a common method adopted in SMLM. But unfortunately, crosstalk appears usually in each detection image, so the discarding will result in low efficiency and prolong the imaging time. Furthermore, for those molecules activated but discarded, more excitations are needed to localize them, so the risk of photobleaching will be increased as well. In this sense, selective imaging is a kind of passive background suppression method. Others, such as activation by two-photon excitation [10,11], and what we proposed in this Letter, can be regarded as active background suppression methods. The key idea of the proposed method is axially selective activation of molecules in SMLM to keep most of activated molecules in a single thin layer, by means of axially modulating the probability of molecules staying at activated state by axially modulated illumination. Fluorophores, such as Cy5 and Alexa 647, that can be reversibly switched between fluorescent and nonfluorescent states with two wavelengths of light are available here. The imaging process here is the same with what is used in dSTORM [5]. Activation light activates molecules from a nonfluorescent state to a fluorescent state, while deactivation light works reversely and serves as the imaging light. Both activation and deactivation lights are assumed to be s polarized.

Illumination intensities of the two axial standing waves generated by the interference of two activation beams and two deactivation beams can be described as [12]

\[
I_{\lambda i} = I_i[1 + r_i \cos[2k_i \cos(\theta_i)z + \varphi_i]],
\]

\[
k_i = 2n_i \pi/\lambda_i, \quad (i = 1, 2),
\]

where \(I_i\) is the intensity constant, \(r_i\) is the contrast which is dependent on the amplitude ratio of the two
interfered beams, \( \theta_i \) is the angle of the beams with respect to the optical axis, \( \lambda_i \) is the wavelength, \( n_i \) is the refractive index, and \( \varphi_i \) is the relative phase difference of the two interfered beams. The suffix \( -i \) indicates that the valuable is activation (\( i = 1 \)) or deactivation (\( i = 2 \)) related.

Activation and deactivation rate constants, \( k_{on} \) and \( k_{off} \), are linearly dependent on the activation and deactivation laser power, respectively, \( k_{on} = \eta \lambda_{1} \), \( k_{off} = \eta \lambda_{2} \), where \( \eta_1 \) and \( \eta_2 \) are two proportional factors. When samples are illuminated with these two standing waves simultaneously, the proportion of fluorescent molecules decays exponentially at the beginning, \( p = \exp(-(k_{on}+k_{off})t) + k_{on}/(k_{on}+k_{off}) \), and then achieves the final equilibrium, \( p = k_{on}/(k_{on}+k_{off}) \). It means that at equilibrium, the probability of molecules staying on the activated state can be calculated from

\[
p = \frac{1}{1 + \frac{\eta_2 \lambda_2 [1 + r_2 \cos(2k_2z + \varphi_2)]}{\eta_1 \lambda_1 [1 + r_1 \cos(2k_1z + \varphi_1)]}}
\]  

(2)

If \( \varphi_i \) are designed as \( \varphi_1 = 0 \) and \( \varphi_2 = \pi \), a zero-point location is defined where activation standing wave peak and deactivation standing wave trough coincide, and \( p \) meets its maximum (\( p_{\text{max}} \)) here, so this peak of \( p \) is called the central peak. Because the activation and deactivation wavelengths are different, the two standing waves have different periods, and the probability \( p \) is not as periodic as the activation standing wave. With the activation standing wave, some peaks of \( p \) will be depressed, and furthermore, because \( p \) is nonlinearly dependent on the ratio of deactivation intensity to activation intensity, those remaining peaks will shrink in width dramatically. The calculated results are illustrated in Fig. 1, based on the reported photoswitching characteristic of Cy5 [5] illuminated with 514 nm (activation) and 647 nm (deactivation) lasers. The ratio of the input activation power to activation power is denoted as \( R \). It is 500, higher than what is used in dSTORM. Actually, power ratio here is axially modulated, while in dSTORM, it is fixed along the \( z \) axis. Nevertheless, in the scheme, \( k_{on} \) and \( k_{off} \) are still linearly proportional to the activation and deactivation intensity, respectively, the proposed method is still feasible.

Until now, fluorescent molecules can be selectively activated in several thin layers. However, the crosstalk from different layers is still a tough problem. As a solution, spatially incoherent monochromatic illumination is introduced, as what is used in I\( \Phi \)M [13]. Now, activation and deactivation illumination intensities along the \( z \) axis are [14]:

\[
P_{\lambda i} = \int_{-\infty}^{\alpha_i} d\theta_i \sin(\theta_i) I_{\lambda i}, \quad (i = 1, 2),
\]

(3)

where \( \alpha_i \) is the maximum angle between the spatially incoherent light and the \( z \) axis. Compared with the previous illumination, the power ratio of deactivation to activation light around the zero point is almost unchanged, while the local power ratio at locations deviated from zero point is not axially modulated anymore. So, correspondingly, the central peak of \( p \) is almost kept, while those noncentered peaks are depressed dramatically. Numerical calculating results shown in Fig. 2(a) demonstrate that those noncentered probability peaks are depressed dramatically to be lower than about 10% of \( p_{\text{max}} \).

The basic requirement for SMLM is that the on-state molecules are further apart with each other than the microscope’s resolution, which means \( p_{\text{max}} \) should be adjustable according to the labeling density. In this scheme, \( p_{\text{max}} \) and the FWHM of the central peak in Fig. 2(a) can be adjusted by the power

\[
\begin{align*}
\text{(a)} & \quad \text{For the same parameters set in Fig. 1, distribution of } p \text{ along the } z \text{ axis in two cases, } \alpha = 0^\circ \text{ and } 60^\circ, \text{ are compared.} \\
\text{(b)} & \quad \text{Plots } p_{\text{max}} \text{ and width (FWHM) of the central peak are generated as functions of the power ratio } R. \\
\text{(c)} & \quad \text{Plot of the percent of the total activated molecules in the central peak as a function of the occupied thickness.}
\end{align*}
\]

![Fig. 1.](image1.png)  
![Fig. 2.](image2.png)
ratio $R$ [see Fig. 2(b)]. Larger $R$ will result in lower $p_{\text{max}}$, while the fraction of activated molecules outside the central layer (the background) is also scaled down. At $R=500$, FWHM of the central peak is about 39 nm. More than 60% of the activated molecules near the zero point are in this region, and about 90% of the activated molecules are located in a 90-nm-thick layer [see Fig. 2(c)]. Contrast $r_i$ can influence $p_{\text{max}}$ and the background suppression efficiency (BSE), which is defined as the intensity ratio of the central peak to the depressed ones, $p_{\text{max}}$/mean, so, accordingly, there is a favorable range for $r_i$. For example, if $p_{\text{max}} < 2.5\%$ and BSE $< 10\%$ are required, and $R$ and $a$ are fixed at 500 and 60°, respectively, $r_i$ should be within 0.83–0.9 [see Fig. 3(a)]. For samples with little scattering, such as a cell, it is feasible. Additionally, spatial incoherence of the activation light is not so important as that of the deactivation light. If deactivation illumination is spatially incoherent, the noncentered peaks are considerably low even if no spatial incoherence is introduced into activation illumination [see Fig. 3(b)]. In brief, for fluorophores switchable reversibly, such as Cy5 and Alexa 647 et al., suitable parameters, such as $R$, $r_i$, and $a_i$ can be properly selected to get a desired distribution of $p$ along the $z$ axis so that most activated molecules are located in a single thin layer with a thickness of a few tens of nanometers, and at the same time, the density of activated molecules in that layer is low enough for SMLM.

The background originated from the specific activation is suppressed by our proposed method. However, there is another kind of background brought about by the blinking fraction, which is the part of molecules nonspecifically activated by the deactivation light itself [9]. This fraction is independent on the intensity of deactivation light, so this kind of background cannot be suppressed by this scheme. However, under proper conditions, this blinking fraction is considerably low [9], which means that $p$ is only slightly higher than what is shown in Fig. 2(a) when this effect is taken into account.

In conclusion, we have reported a method of suppressing background for SMLM by axially selective activation of molecules. Numerical calculation results based on the known photoswitching characteristic of Cy5 demonstrated that for molecules along the $z$ axis, the probability of being activated has a single peak with FWHM of only 39 nm, and about 90% of the activated molecules are located in a 90-nm-thick layer around zero point. This selective activation method, combined with those axial localization methods [6–9], can be used in 3D nanoimaging.

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References