Analysis of Lateral Resolution Improvement for Fluorescence Microscopy using Standing Evanescent Light

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Abstract. This paper presents a lateral resolution improvement for TIRF (Total Internal Reflection Fluorescence) microscopy that employs combined use of standing evanescent light and scattering distribution retrieval algorithm with successive approximation. In this method, the variations of fluorescence images are detected by shifting the standing evanescent light with nanometer scale. Then the lateral resolution improvement beyond the diffraction limit can be expected by using the multiple fluorescence images, because the variations of which include the high frequency components of the specimen on the TIR interface. The theoretical analysis based on the Fourier optics and the computer simulations suggest that the proposed method has about twice as high resolution as conventional optical microscopy.

Introduction

Optical microscopy is still a useful metrology technique to observe the magnified image. Because light has intrinsic characteristics of low energy and spatial transmission, no damage is given to samples and high throughput is achievable by remote imaging. But the major limitation of optical microscopy is the diffraction limit in resolution, which is about half the wavelength. Various types of optical microscopies have been developed so far to overcome the diffraction limit, such as confocal microscopy [1], SNOM [2], TIR fluorescence microscopy [3]. In this research, we are developing a TIRF (Total Internal Reflection Fluorescence) microscopy that employs combined use of standing evanescent light [4],[5] and scattering distribution retrieval algorithm with successive approximation [5].

In this paper, the improvement of lateral resolution is derived by taking the frequency of the standing evanescent light illumination into consideration. And in order to verify the feasibility of this proposed method, the theoretical analyses were carried out by computer simulation.

TIRF Microscopy using Standing Evanescent Light Illumination

Figure 1 shows a schematic diagram of TIRF microscopic system using standing evanescent light illumination. Standing evanescent light is formed on the interface of total internal reflection by a superposition of an incident light and a counter propagating incident light. Illuminate samples are confined within several hundred nanometers from the TIR interface because the intensity of evanescent light decreases exponentially in a vertical direction. The standing evanescent light has high-frequency modulation in a lateral direction, whose peak-to-peak distance is about half the wavelength (several hundred nanometers). The illuminated samples emit fluorescence light and its distribution is magnified and detected through far-field imaging optics. And the microscopic image of the fluorescence light distribution $r(x)$ can be expressed as:

$$r(x) = psf(x) \otimes (a(x) \cdot i(x)), \tag{1}$$

where, $psf(x)$ is a point-spread function of the imaging optics, $a(x)$ is the fluorescence sample distribution on the TIR surface, and $i(x)$ is the standing evanescent light illumination distribution.

This far-field optical imaging of the fluorescence light distribution $r(x)$ is dominated by the diffraction limit. Because $psf(x)$ acts as a low pass filter in the equation (1), high-frequency information of fluorescence sample distribution $a(x)$ is lost in the far-field optical image $r(x)$ through the imaging optics.

In this method, the multiple fluorescence images of $r(x)$ can be detected by shifting the standing evanescent light with nanometer scale. Since the multiple images of $r(x)$ and the corresponding illumination distributions of $i(x)$ are known, we can reconstruct $a(x)$ with higher frequency by using successive approximation [5] based on the equation (1). Thus we expect to achieve super resolution beyond the diffraction limit.

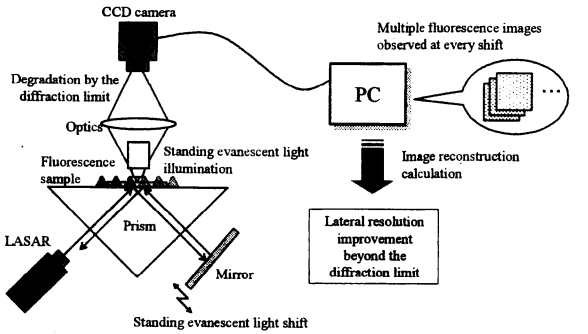


Fig. 1 Concept of TIRF microscopy using standing evanescent light

Lateral Resolution Improvement by Standing Evanescent Light Illumination

In this method, the lateral resolution is improved by the spectrum of the illumination distribution. The standing evanescent light illumination distribution $i(x)$ can be expressed as follows:

$$i(x) = \frac{1 + \cos(2\pi f_m x + \theta)}{2}, \tag{2}$$

where, f_m is the frequency of the standing evanescent light and θ is the phase of the standing evanescent light shift. From equation (1) and equation (2), we have that

$$R(f) = \frac{1}{2} OTF(f) A(f) + \frac{1}{4} e^{i\theta} OTF(f) A(f - f_m) + \frac{1}{4} e^{-i\theta} OTF(f) A(f + f_m), \tag{3}$$

where, $R(f)$ is the Fourier transform of the microscopic image of the fluorescence light distribution $r(x)$, OTF is the optical transfer function, and $A(f)$ is the Fourier transform of the fluorescence sample distribution on the TIR surface. The first term of this equation means that the spatial frequency of $r(x)$ is restricted by the cutoff frequency f_c depending on the OTF, which is the same as the conventional microscopic system. And the second and third terms of this equation mean that the spatial frequency of $r(x)$ is expanded to the frequency of the standing evanescent light $(f_c \pm f_m)$. Then, we obtain the lateral resolution of proposed optical system. Based on the Rayleigh criterion, the lateral

resolution improved by the effectiveness of the high frequency components of the standing evanescent light distribution can be expressed as:

$$lateral\_resolution = \frac{1.22}{f_c + f_m} = \frac{1.22}{\frac{2N.A.}{\lambda} + \frac{1}{T}}, \quad (4)$$

where, the *N.A.* is the numerical aperture of the imaging optics, λ is the wavelength of the fluorescence light of the sample, and T is the peak-to-peak distance of the standing evanescent light distribution. This equation means that the lateral resolution limit depends on the standing evanescent light distribution as well as the imaging optics. The relationship between the lateral resolution and the peak-to-peak distance of the standing evanescent light distribution T can be expressed as the line graph shown in figure 2. This figure is represented by the equation (4) under the condition that λ is 447nm and N.A. is 1.2. As shown in this figure, the lateral resolution is improved with a decrease in the peak-to-peak distance of the standing light distribution T.

Figure 3 shows the relationship between the lateral resolution and the numerical aperture of the imaging optics *N.A.* under the condition of the peak-to-peak distance $T = 85$ nm. The proposed method has about twice as high resolution as conventional optical microscopy. And the proposed method remains nearly unaffected by the numerical aperture, while the conventional optical microscopic system is mainly affected by the numerical aperture. This fact allows us to observe the high magnified image with long working distance.

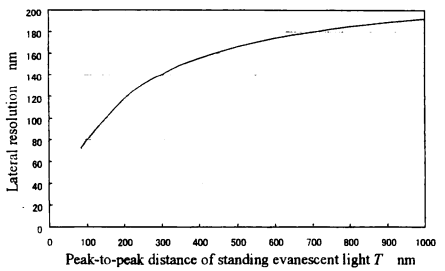


Fig. 2 Relationship between Lateral resolution and peak-to-peak ditance of standing evanescent light T

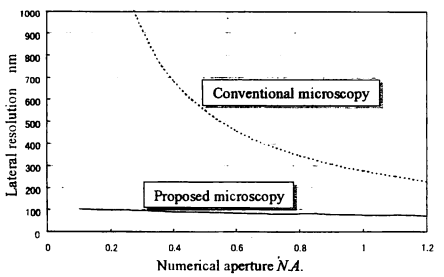
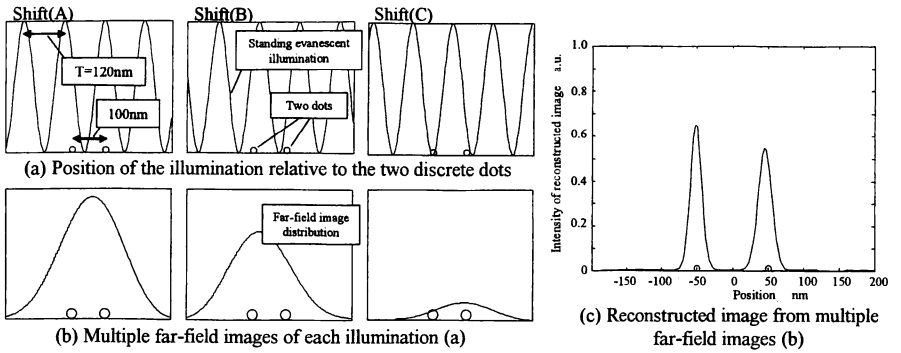


Fig. 3 Relationship between Lateral resolution and numerical aperture *N.A.*

Analysis of Lateral Resolution Improvement by Computer Simulation

In order to verify the lateral resolution improvement of the proposed method, computer simulations were performed. Figure 4 shows the typical simulation result by this method. In this case, two discrete dots sample separated by 100nm was employed as the fluorescence sample on the TIR surface under the condition that *N.A.* = 1.2, $T = 120$nm, $\lambda = 447$ nm. Figure 4(a) shows the relationship between the position of the two dots sample and the standing evanescent light illumination. Figure 4(b) indicates the multiple far-field optical images of two dots sample with the position of figure 4(a), respectively. And figure 4(c) is a reconstructed super resolution image calculated from the multiple far-field optical images of figure 4(b). Figure 4(b) almost corresponds to the conventional microscopic image. These are no longer resolvable because the separations are smaller than the diffraction limit of 227nm. But as shown in figure 4(c), we can resolve the separated two dots beyond the diffraction limit clearly based on the equation (1).



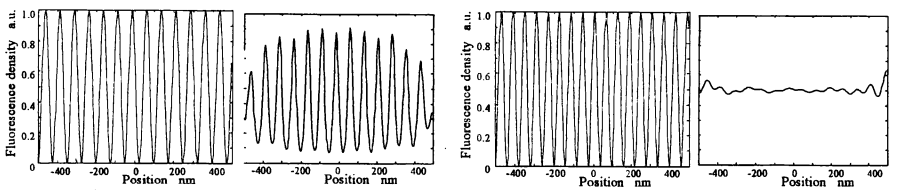
(a) Position of the illumination relative to the two discrete dots

(b) Multiple far-field images of each illumination (a)

(c) Reconstructed image from multiple far-field images (b)

Fig. 4 Typical simulation result of lateral resolution improvement by proposed method (100nm separated dots)

In order to verify the validity of equation (4), we performed another simulation for resolving the sinusoidal continuous variations as the fluorescence sample. Figure 5(a) and (b) show the results at pitch of the sinusoidal variations of 75nm and 60nm respectively under the condition that λ = 447nm, N.A. = 1.2, and T = 104nm. The sinusoidal variations of 60nm could not be resolved, but ones of 75nm could be almost resolved. Under this condition, the lateral resolution represented in equation (4) is calculated to be 81.4nm. Then these results show almost good agreement with the equation (4).



(a) Pitch of sinusoidal validations of 75 nm

(b) Pitch of sinusoidal validations of 60 nm

Fig. 5 Simulation for resolving the sinusoidal continuous variations as fluorescence sample

Conclusions

The lateral resolution of the fluorescence microscopy using standing evanescent light was theoretically analyzed and the equation of the improvement of lateral resolution was obtained by taking the frequency of the standing evanescent light illumination into consideration. This equation suggests that the proposed method has about twice as high resolution as conventional optical microscopy.

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