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Measurement of point-spread function (PSF) for confocal fluorescence microscopy

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ABSTRACT

In this paper, we describe size-effect of fluorescent microsphere for measuring point-spread function (PSF) in confocal fluorescence microscopy. We present the numerical results for the practically available microsphere size range for measuring PSF, and demonstrate with experiment. Also, the effective PSF is restored with deconvolution technique within an acceptable error. Also the size-effect for measuring phase modulated PSF, which has two vicinal peaks, is described. The numerical and experimental result is also presented.

Keywords: Confocal microscopy, PSF, Deconvolution, phase modulation

1. INTRODUCTION

There has been a tremendous explosion in the popularity of confocal laser scanning microscopy (CLSM) in recent years, due in part to the relative ease with which extremely high-quality images can be obtained from specimens. A growing number of CLSM applications in cell biology and medicine rely on imaging both fixed and living cells and tissues, and predominantly employ fluorescence. Confocal microscopy offers several advantages over conventional widefield optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane, and the capability to collect serial optical sections from thick specimens[1].

An image measured by optical microscopy is convoluted image of PSF and specimen. It means smaller PSF makes better image quality for optical microscopy. The diffraction phenomenon, however, limits effective PSF, which limits the resolution of the optical microscopy[2]. The several researches were performed to improve spatial and axial resolution: such as two-photon microscopy, 4-pi confocal microscopy, STED (Stimulated Emission Depletion) microscopy, structured illumination microscopy, and so on. PSF (Point Spread Function) engineering technique achieves superior resolution by changing spot shape. The resolution can be further improved by image restoration technique, like

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deconvolution[3]. An image resolution could be improved applying the deconvolution or filtering technique[4]. But, the accuracy of expected PSF of optical microscope is an important factor applying deconvolution.

The microscope's PSF is usually measured by imaging a small fluorescent bead. The smaller microsphere could reflect PSF more exactly. But, there is a tradeoff in this measurement: very small microspheres are dim and bleach rapidly, while larger microspheres are a poorer approximation to a point source[5].

In this paper, we present the results about the measurement the PSF of microscope. We described about the size-effect for measuring PSF. The deconvolution is applied to remove the effect of fluorescent microsphere, and the restored PSF shows reliable result. Also the size-effect for measuring phase modulated PSF, which has two vicinal peaks, is described. The numerical and experimental result is also presented.

2. SYSTEM CONFIGURATION

The confocal fluorescence microscopy is designed for the measurement of PSF. The system is based on the commercial upright microscope body (Olympus), and the other optical component is arranged for the fluorescence confocal microscopy. The configuration of the confocal fluorescence microscopy is schematically shown in Figure 1.

The HeNe laser (Coherent, 543.8nm) is used as a light source, and laser output is directly coupled to single-mode optical fiber with focusing lens. The optical fiber flexibly delivers the laser to the system and collimated with collimating lens. Also, the optical fiber and collimating lens acts as a beam expander and a point source. The adjusting aperture makes appropriate beam-size and laser beam is go through dichroic mirror-set via folding mirror. On the beam path, the phase

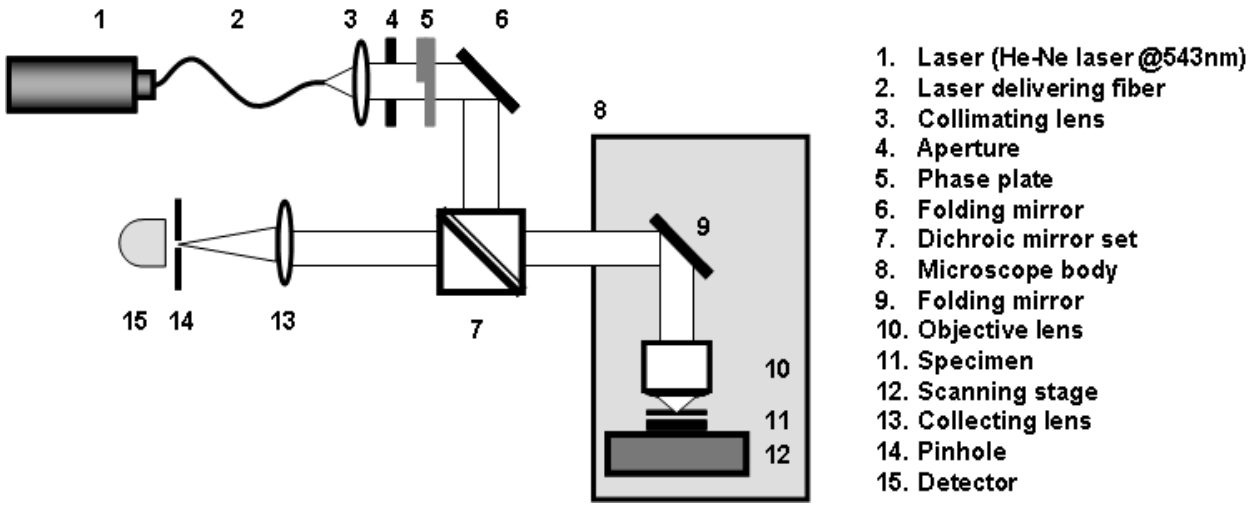

Figure 1: The configuration of confocal fluorescence microscopy for the measurement of PSF. The phase plate is adopted to partially modulate the phase of laser. The commercial upright microscopy and precision nanopositioning stage is adopted. The fluorescent light intensity is measured in avalanche photodiode (APD).

plate is adopted which partially modulates the phase of laser beam. The details of phase modulation are described in section 3.

We adopted commercial upright microscope (Olympus, BX51) for the convenience of operation. It allows observing wide-field image of a specimen through objective lens and eyepiece. The external side-port and folding mirror are added to accept excitation laser beam for the use of confocal microscopy. Excitation laser beam is heading objective lens via folding mirror with a proper beam alignment. The objective lens (Olympus, 100X/NA1.3, Oil) is used to focus excitation laser beam and to collect emission fluorescent light.

The commercially available fluorescent microspheres (Molecular probes, Fluosphere) are used for the PSF measurement. The microspheres are stained in the slide glass. The microspheres absorb a light at 560nm and emit the light of 580nm wavelength. Various size of microsphere is used to investigate the effect of size on PSF measurement. The high resolution nanopositioning stage (Piezojena, TRITOR 100) is used for 3-dimensional scanning. The stage has 80μm stroke and 1.6nm resolution for the closed loop operation.

When excitation laser is focused on the specimen, the fluorescence emission is gathered by the objective lens. The collected emission light go back through folding mirror to pass diciroic mirror set, which contains emission filter for the proper emission wavelength. The emission light is focused with collecting lens and finally detected by avalanche photodiode (APD; Hamamatsu, C5460) through the pinhole to prevent the light from out of focus plane.

3. PSF MEASUREMENT

An image measured by optical microscopy is convoluted image of PSF and specimen. If we know PSF, measured image can be restored by removing PSF effect using deconvolution technique. The microscope's PSF is usually measured by imaging a small fluorescent microsphere. The small fluorescent microsphere, however, still has a finite size, blurring PSF is inevitable. In this section, various size of fluorescent microsphere is used to calculate PSF theoretically and experimentally. The numerical evaluation of the PSF is derived from the diffraction theory, and direct experimental measurement using a small fluorescent microsphere. Also, deconvolution technique is used to restore original PSF.

3.1 Simulation of PSF

An image measured by optical microscopy is convoluted image of PSF and specimen. The convolution of theoretical PSF (for 100X 1.3NA oil immersion lens) and fluorescent microsphere, which is an image optical microscopy can present, is calculated and theoretical PSF is restored using deconvolution technique. The Lucy Richard maximum likelihood estimation algorithm is used for the PSF deconvolution. The calculated XY PSF is presented in figure2. The theoretical PSF calculation is based on geometrical ray tracing and diffraction theory. The diameter of the fluorescent microspheres is 0.17μm, 0.5μm, and 1μm.

Cross-section graph is presented in figure 3. Bigger microsphere has bigger convoluted PSF, but restored PSF is very similar to theoretical PSF. If microsphere is real-point like source, convoluted PSF is same to theoretical PSF. It's finite

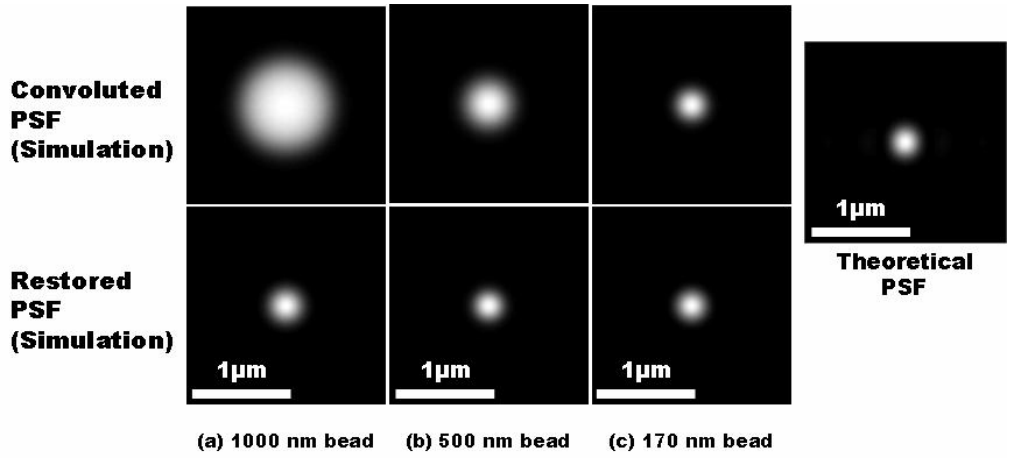

Figure 2: Simulation of XY PSF. Top line is convoluted PSF of theoretical PSF and various fluorescent microspheres (a) 1000nm (b) 500nm (c) 170nm. Bottom line is restored PSF using deconvolution technique. Theoretical PSF is comparable on right of figure. Each image size is 2×2μm.

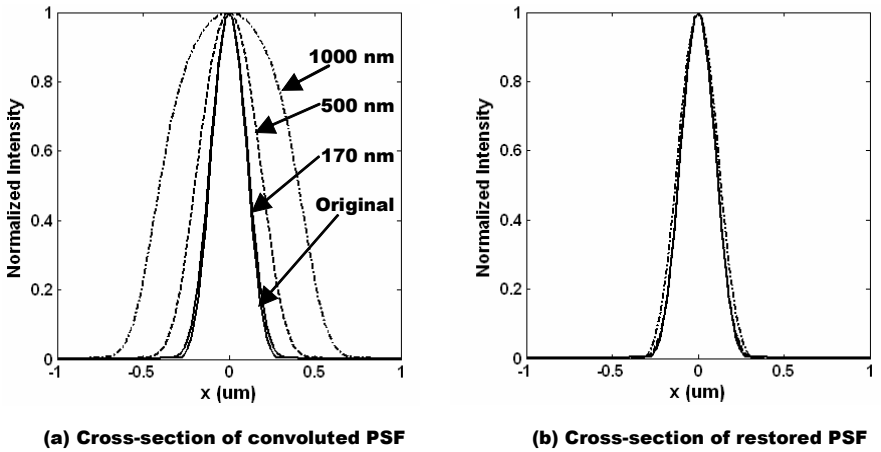

Figure 3: Cross-section of (a) convoluted PSF (b) restored PSF. Intensity is normalized for each microsphere size.

size, however, need a compromise. As a compromise a microsphere diameter about one-third of the resolution size limit expected for the microscope objective in use should be chosen. Considering Rayleigh resolution limit, recommended microsphere size is $0.41\lambda/NA$[5]. In our system, recommended size is about 171nm (λ=543.8nm, NA=1.3). The convoluted PSF with 170nm microsphere is almost same to original PSF in figure 3(a). The restored PSFs show similar sidelobe to original PSF for the 170nm and 500nm microsphere. The FWHM is a little increased for the 1μm microsphere, but it is still acceptable.

3.2 Measurement of PSF

The stage-scanned confocal fluorescent microscopy in figure 1 is used to measure PSF. The diameter of the fluorescent microspheres is 0.17μm, 0.5μm, and 1μm (same as to simulation), which is commercially available from Molecular

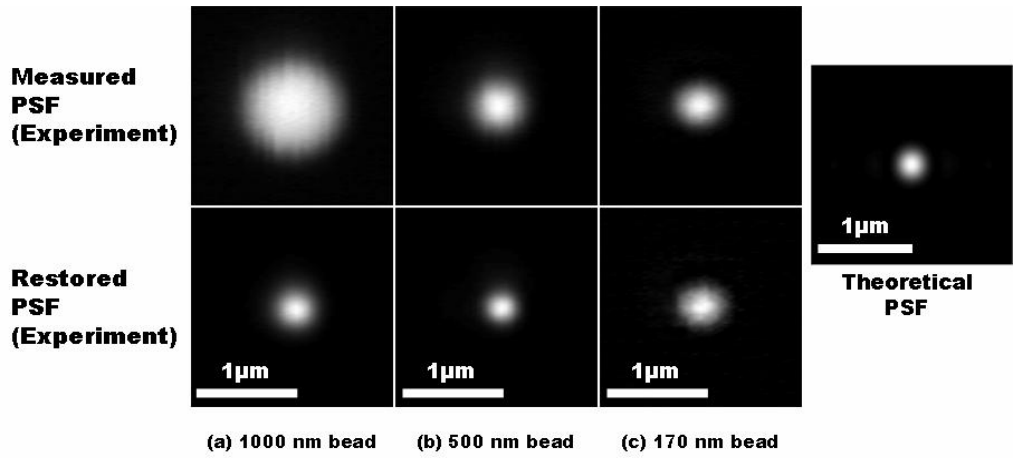

Figure 4: Measured PSF. Top line is measured PSF experimentally for various microspheres (a) 1000nm (b) 500nm (c) 170nm. Bottom line is restored PSF using deconvolution technique.

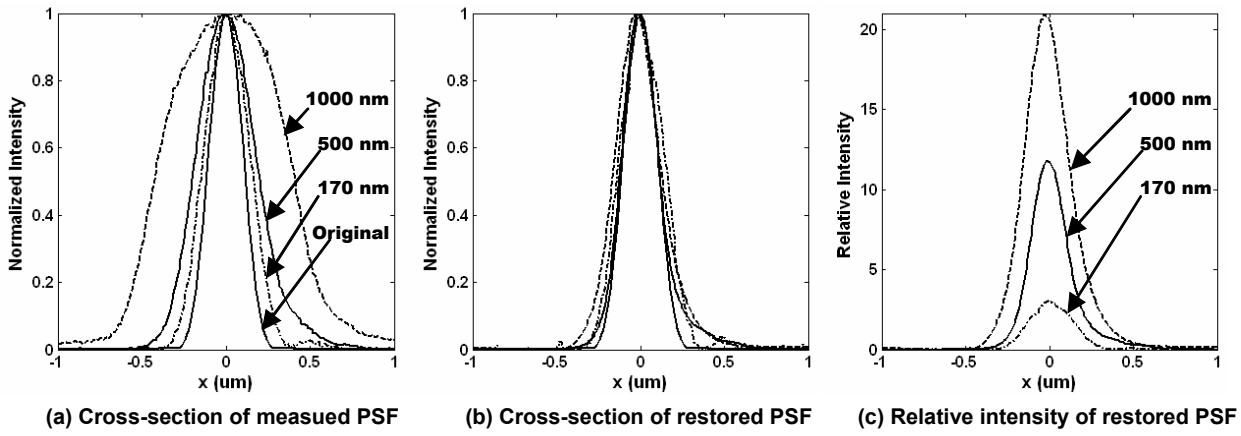

Figure 5: Cross-section of (a) measured PSF (b) restored PSF. (c) is plot of relative intensity of restored PSF. Original PSF is numerical result from figure 3.

Probes. In figure 4, measured PSF shows that smaller microsphere is closer to original PSF. But measured PSF size increases with microsphere size. The restored PSF is also very similar to theoretical PSF. It is a little bigger than theoretical PSF for 1μm microsphere.

The restored PSF for 170nm looks bigger than other two microspheres, because its signal-to-noise ratio (SNR) is weak than other two cases. This is comparable in figure 5(c): bigger microsphere shows better SNR. There is another possibility that 500nm microsphere size is actually smaller than 500nm. If we assume that microsphere is bigger than its real-size, restored PSF can be smaller than its real PSF. To verify this situation FWHM is calculated from figure 5(b) and listed in table 1. In table, for the case of 450nm, the measured PSF of 500nm microsphere is restored with 450nm microsphere assumption. FWHM is almost similar except for the case of 500nm. Note that instead of 500nm microsphere, when we assume microsphere real-size is 450nm FWHM is very similar with other cases. Another experimental evidence for assuming 450nm microsphere is in next section. With 450nm microsphere assumption,

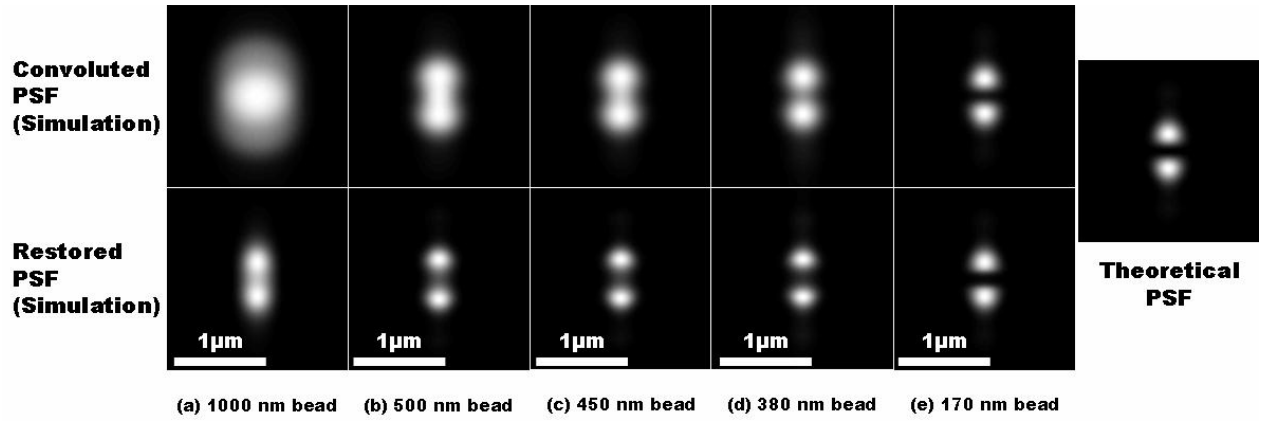

Figure 6: Simulation of phase modulated XY PSF. Theoretical PSF is on the right of figure. Phase modulation makes the two vicinal peaks instead of single peak. Top line is convoluted PSF of theoretical PSF and various fluorescent microspheres (a) 1000nm (b) 500nm (c) 450nm (d) 380nm (e) 170nm. Bottom line is restored PSF.

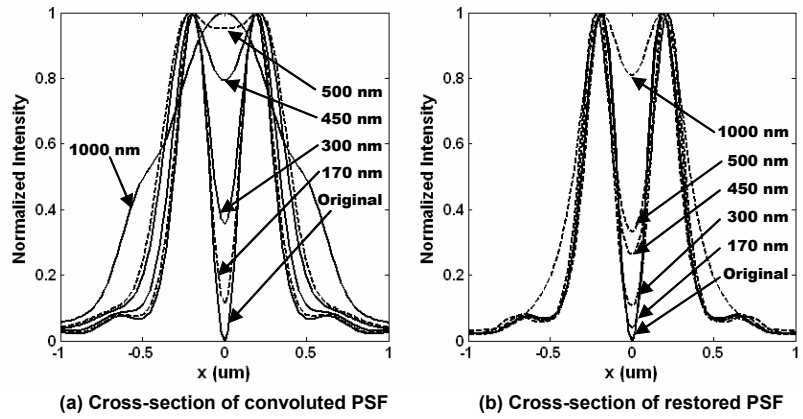

Figure 7: Cross-section of (a) convoluted PSF (b) restored PSF.

FWHM is within error range (±10nm). There are some error sources such as defocusing, photo-bleaching effect, and aberrations.

3.3 PSF measurement with phase modulation

The phase modulation device is made with optical glass (BK 7) partially coated with MgF_2. The optical path length of coated part is different to uncoated part. For this experiment, the upper-half of circular glass is non-coated, and lower

Table 1: FWHM of restored PSF in figure 5(b). It is calculated from interpolation of measured data Calculation accuracy is ±10nm.

Microsphere size	Theoretical	170nm	450nm (Assumed)	500nm	1000nm
FWHM	230 ±10nm	290 ±10nm	280 ±10nm	250 ±10nm	300 ±10nm

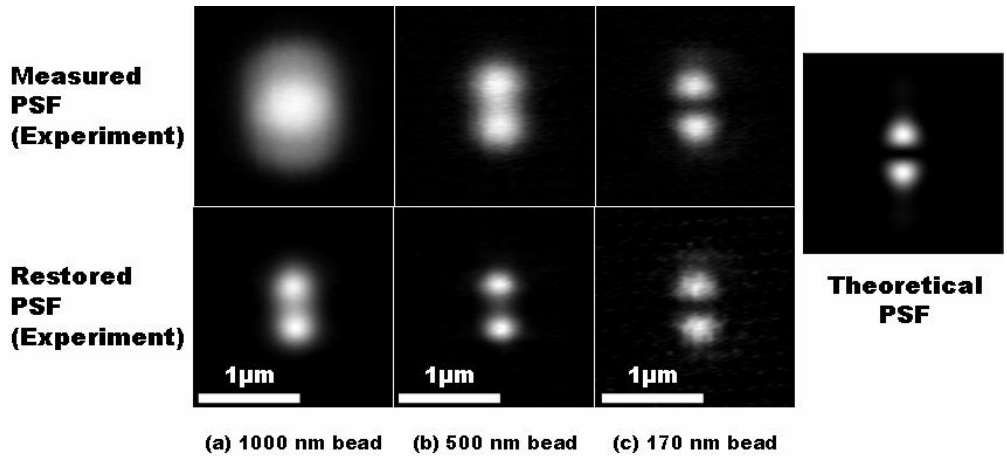

Figure 8: Measured PSF with phase modulation. Top line is measured PSF experimentally for various microspheres (a) 1000nm (b) 500nm (c) 170nm. Bottom line is restored PSF using deconvolution technique.

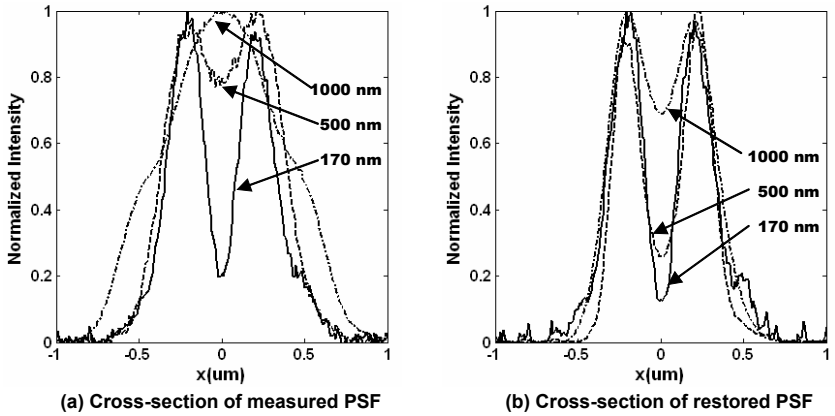

Figure 9: Cross-section of PSF with phase modulation in experiment. (a) Measured PSF (b) restored PSF.

half is coated with MgF_2 (*n=1.382*). The coating thickness is controlled so that upper and lower half has the phase difference of π. The phase modulation makes two vicinal peaks at the focal spot. It is from the interference effect of asymmetric phase distribution. The numerical simulation of the phase modulated XY PSF is presented in figure 6. Many kind of modulation pattern can be considered and other phase modulation technique can be used[6].

The modulated PSF numerically is convoluted with various sizes of microsphere. And restored PSF is calculated using deconvolution technique as in section 3.1. In figure 6, for bigger size of microsphere, two vicinal peaks are fainter. Comparing restored PSF, when microsphere size is 170nm, the restored PSF has similar shape and size. For the 500nm microsphere, vicinal peaks are distinguishable but it shape and central minimum value is not restored. For the 1μm microsphere, two peaks area not distinguishable.

The cross-section graphs of PSF are clearly showing the size effect for measuring PSF in figure 7(b). Note that the central minimum value between two vicinal peaks is closer to zero for the smaller microsphere. Getting higher the value of central valley, it is hard to distinguish two vicinal peaks: PSF images are blurring for bigger microsphere in figure 6. It

Table 2: Contrast in central Peak-to-Valley of restored PSF in figure 7. Contrast is defined as the ratio of signal difference (of peak-to-valley) to mean value

Microsphere size	Theoretical	170nm	300nm	380nm	400	450nm	500nm	1000nm
Contrast (%)	100	96	88	80	78	71	63	15

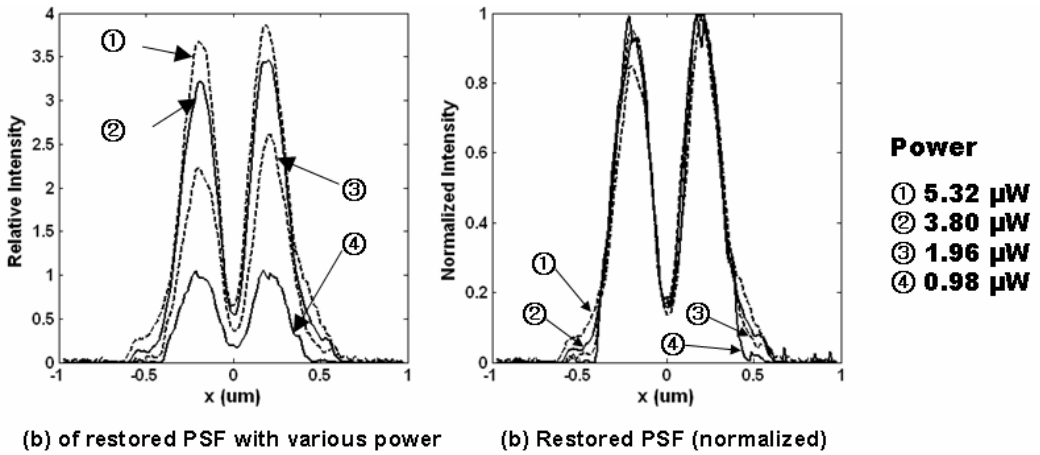

Figure 10: (a) Measured PSF with phase modulation for the various excitation powers. (b) Normalized with maximum intensity.

means restored PSF does not reflect original PSF perfectly.

To make some criteria for allowable microsphere size, the contrast between peak and valley is calculated. In the calculation, contrast is defined as the ratio of signal difference (of peak-to-valley) to mean value. Table 2 shows the calculated contrast for the various micro-sphere sizes. If the contrast of 30% is allowed, about acceptable size of microsphere is about 450nm. If 20% contrast is allowable, microsphere size should be less than 380nm, theoretically.

Figure 8 shows measured PSF with phase modulation and restored PSF, experimentally. For the 170nm microsphere, shape and size is most similar to theoretical PSF. Also, its central valley is well restored. But its poor SNR makes poor image quality. Increasing the size of microsphere, two vicinal peaks are faint and central valley is blurred.

The cross-sectional graph in figure 9 clearly shows the central minimum value. The experimental tendency is similar to the numerical simulation in figure 7. But, its central minimum is higher than simulation on the whole. For the case of 500nm microsphere, the restored PSF is smaller than the others. It may be caused form different size information as mentioned in section 3.2. Comparing figure 9 with figure 7, the actual size of 500nm microsphere may smaller than 450nm. The 500nm microsphere will be measured with appropriate microscopy (such as SEM or TEM) soon.

Figure 10 shows the relation of measured PSF and laser power. PSF is measured with various laser powers in same microsphere (170nm). Increasing power makes higher fluorescent intensity, but high power excitation would be limited by photo-bleaching effect. The phto-bleaching is also affected by exposure time. In figure 10, when power is 5.32μW,

there was some photo-bleaching already. Although excitation power is different, the normalized PSF profile is very similar. But small excitation power could have low SNR, and high excitation power have photo-bleaching.

4. CONCLUSION

The point-spread function is the most important parameter of optical microscopy. A PSF is usually measured by imaging a small fluorescent microsphere. A smaller microsphere can measure PSF more accurately. But, there is a tradeoff in this measurement: very small microspheres are dim and bleach rapidly, while larger microspheres are a poorer approximation to a point source. The deconvolution technique effectively restores original PSF when appropriate size of microsphere is used. The available size of fluorescent microsphere for measuring vicinal two peaks is shown in numerically and experimentally. When the size of microsphere is not small enough, the central minim of valley cannot well be restored even with deconvolution technique.

ACKNOWLEDGEMENT

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