Improvement of detected intensity in confocal microscopy by using reflecting optical system

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In confocal microscopy, the level of the detected intensity is low, which reduces the contrast of the images. We propose a reflecting optical system to improve the detected intensity. The result of the optimal design is presented and the performance of the resulting reflecting optical system is evaluated. A numerical simulation of the effect of the reflecting optical system on the total system performance is also presented. A standard specimen is imaged to show the benefits of the proposed reflecting optical system. The results in this Note demonstrate a 37% increase of the detected intensity and better contrast of the image. © 2004 American Institute of Physics.

Confocal microscopy was invented by Minsky, and has become an essential measurement tool in many fields.1,2 Due to its ability of optical sectioning and higher resolution, confocal microscopy obtains clearer images than any conventional optical microscopy has ever obtained, and three-dimensional images of organelles in the cells can be obtained only by confocal microscopy.

However, confocal microscopy has some shortcomings. One of them is the low level of detected intensity. Due to the aperture pinhole and complex optical systems, only a small portion of the emitted photons can arrive at the photon detector. According to David R. Sandison’s work,1 only 0.02% of the emitted photons are detected in commercial confocal microscopy, MRC-600 (BioRad). In some cases, this low detected signal is quite comparable to the noise signal. Low signal-to-noise ratio ruins imaging performance, especially the contrast of the images.

A few methods have been proposed to increase the detected intensity. Folded optics were used to increase the detected intensity by three or four times using a second objective and a plane mirror.1,4 4Pi-confocal microscopy provided not only better resolution, but also better image contrast.3 This enhanced contrast was mainly due to the increase of the detected intensity achieved by symmetric optics in 4Pi-confocal microscopy.

No research has been done on the method to increase the detected intensity in beam scanning confocal microscopy. Beam scanning confocal microscopy is widely used in commercial systems, since images can be acquired in nearly real time and specimens under inspection are free of vibration caused by the movement of the stage. However, optics in the beam scanning system are complicated, which makes it difficult to use folded optics or the symmetric optics mentioned in the earlier paragraph.

In this Note, we propose a reflecting optical system in beam scanning confocal microscopy to improve the detected intensity. We describe the conceptual design of the proposed optics and optimal design results. We also discuss the possible degradation of the performance induced by the specimen. Experimental results are presented at the end of the Note.

A schematic of the proposed reflecting optical system is illustrated in Fig. 1. The first objective lens focuses the beam from the scanning optics onto the specimen. The fluorescence beam from the illuminated focal point is recollimated by the second objective lens in the reflecting optical system. The spherical lens focuses the recollimated beam onto the spherical mirror. Since the surface of the spherical mirror is conjugate to the scanning surface, the reflected beam from the spherical mirror is focused onto the scanning point. This refocused beam passes through the first objective lens and enters the scanning optics. Therefore, the photon detector is able to detect not only the fluorescence beam propagating backward, but also the fluorescence beam propagating forward, which enhances the detected intensity. The fluorescence beam propagating forward is detected regardless of the position of the scanning point, which makes the reflecting optical system applicable in beam scanning confocal microscopy.

The main function of the reflecting optical system is to focus the fluorescence beam propagating forward onto the scanning point. Since the spherical surfaces used in the re-

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reflecting optical system inherently have aberrations, the performance can be ruined by a mismatched combination of the components. To obtain the best performance, optimal design is necessary. In the optimal design process, the objective function should be defined. We used the finite ray tracing method to calculate the incidence positions and angles of the reflected fluorescence beams onto the back aperture plane of the second objective lens. The objective function was defined as a sum of the deviation of the incidence position of the marginal ray in the reflected beam from the aperture of the second objective lens and the converging angle of two rays in the reflected beam entering the second objective lens. The self-coded MATLAB (Mathwork, Natik, MA) optimal design program was used to minimize the objective function. We considered spherical lenses and spherical mirrors from Newport (Irvine, CA) as candidates for the components in the reflecting optical system. As a result of the optimal design, spherical lens, KBX079, and spherical mirror, 10DC500, were selected, and the optimal distance between each component was calculated.

We evaluated the result of the optimal design using the commercial optical simulation program OSLO (Lambda Research, Littleton, MA). Wave front error on the surface of the spherical mirror without the specimen was calculated. When the scanning point is on the optical axis, rms wave front error was 0.001322 wave. rms wave front error was 0.001461 wave even at the edge of the view field. According to the Rayleigh criteria, nearly diffraction-limited performance is achievable, when rms wave front is less than 0.05 wave. In the reflecting optical system, rms wave front error was less than 0.003 wave for the round optical path. This shows that the reflecting optical system obtains diffraction-limited performance, when there is no specimen.

When there is a specimen between the two objective lenses, the refractive-index-mismatched specimen reduces the level of the detected signal and resolution. A few studies have been done on the role of the specimen-induced degradation of the performance. Here we consider the effect of a refractive-index-mismatched specimen on the performance of the reflecting optical system by evaluating the rms wave front error on the surface of the spherical mirror (Fig. 2). The specimen was assumed to be aqueous \( n_3 = 1.33 \) and placed between two 170-\( \mu \)m-thick glass coverslips \( n_2 = 1.54 \). The immersion medium was assumed to be air \( n_1 = 1.00 \). The rms wave front error is plotted against the thickness of the specimen in Fig. 3(a). The rms wave front errors were evaluated for different numerical apertures (NA s) of the objective.

![FIG. 1. Conceptual design of reflecting optical system. (A)' and (B)' are conjugate points to (A) and (B).](image1)

![FIG. 2. Geometry and notation used for the numerical calculations.](image2)

![FIG. 3. The rms wavefront error against the thickness of the specimen: (a) without the autofocusing, and (b) with autofocusing.](image3)
lenses ranging from 0.4 to 0.8. Since rms wave front errors for NAs over 0.7 were much over the 0.025 wave, even when the thickness of the specimen is 0, the simulation results for NAs over 0.7 are not plotted. According to the Rayleigh criteria about the wave front error, the rms wave front error on the surface of the spherical mirror should be less than 0.025 wave. As shown in Fig. 3(a), the reflecting optical system is diffraction limited, when the specimen thickness is less than 30 and 13 μm for NA of 0.4 and 0.6. For only small NA objective lenses, the reflecting optical system obtains diffraction-limited performance for biological specimens, whose thickness is on the order of several tens of μm. We also evaluated the rms wave front error as we changed the location of the focal point in the specimen, but the effect of the focal point location on the rms wave front error was negligible.

The autofocusing of the second objective lens in the reflecting optical system can compensate for the aberration and the displacement of the focal point induced by the specimen. We calculated the rms wave front error when autofocusing was used. The optimization tool in OSLO was used to calculate the position of the second objective lens that minimized the rms wave front error. Figure 3(b) shows the rms wave front error as a function of the thickness of the specimen. The reflecting optical system obtains diffraction-limited performance for an over 350-μm-thick biological specimen even with the 0.7 NA objective lens. The amount of the objective lens movement was about 100 μm and the necessary positioning accuracy was of the order of several ten nm. This requirement can be fulfilled with precision actuators, such as Picomotor (New Focus, San Jose, CA). However, the optical probe is distorted as the probe depth increases. This imposes a real limitation on the possible thickness of the specimen. As can be seen from the discussion above, the allowable thickness of the specimen is limited, but not by the reflecting optical system, when autofocusing is used.

We integrated the reflecting optical system on a self-made beam scanning confocal microscope. The self-made beam scanning confocal microscope was composed of two galvanomirrors (Cambridge Technology, Cambridge, MA), and the self-made scanning optics. A 633 nm He–Ne laser (JDS Uniphase, San Jose, CA) was used as a light source.

We used the Focalcheck microsphere (Molecular Probes, Eugene, OR) to show the performance of the reflecting optical system. The experimental results with and without the reflecting optical system are shown in Fig. 4. As can be shown from Figs. 4(a) and 4(b), the image obtained with the reflecting optical system has better contrast. The line profile of section A–A' shows the improvement of the detected intensity when the reflecting optical system is used [Fig. 4(c)]. The experimental results showed that the detected intensity increased about 37%. In the ideal case, the amount of the increase should be 100%. Since we used a 0.6 NA objective lens as the first objective lens, and a 0.4 NA objective lens as the second objective lens in the reflecting optical system, the amount of the increase was limited by the aperture of the second objective lens. The analytical approach showed that the amount of the energy captured by the objective lens with an aperture angle of α is proportional to (1 − cos α). Therefore, the energy captured by the 0.4 NA objective lens is 42% of the energy captured by the 0.6 NA objective lens. However, the increase of the experiment, 37% of increase, was slightly less than what is expected by the theoretical analysis; 42% of increase. This is explainable by the reflection at each coverslip and the scattering effect of the specimen.

In this Note, we have proposed a reflecting optical system to improve the detected intensity in beam scanning confocal microscopy. The numerical analysis shows that the reflecting optical system does not impose any harmful effect on the total system performance when a relatively low NA objective lens is used and autofocusing is provided. The experimental results showed improvement of the detected intensity and enhanced contrast of the images. The optical path length of the reflecting optical system can be minimized by using more optical components, and the resulting reflecting optical system can be made as a separate module compatible with commercial beam scanning confocal microscopes.