Characterization of gradient-index lens-fiber spacing toward applications in two-photon fluorescence endoscopy

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We report on the experimental investigation into the characterization of two-photon fluorescence microscopy based on the separation distance of a single-mode optical fiber coupler and a gradient-index (GRIN) rod lens. The collected two-photon fluorescence signal exhibits a maximum intensity at a defined separation distance (gap length) where the increasing effective excitation numerical aperture is balanced by the decreasing confocal emission collection. A maximum signal is found at gap lengths of approximately 2, 1.25, and 1.75 mm for GRIN lenses with pitches of 0.23, 0.25, and 0.29 wavelength at 830 nm. The maximum two-photon fluorescence signal collected corresponds to a threefold reduction of axial resolution (38.5 μm at 1.25 mm), compared with the maximum resolution (11.6 μm at 5.5 mm), as shown by the three-dimensional imaging of 10 μm beads. These results demonstrate an intrinsic trade-off between signal collection and axial resolution.

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1. Introduction

Two-photon microscopy has been widely used as the best noninvasive means of fluorescence microscopy for three-dimensional imaging in thick tissue[1] and in live animals.[2] The advantages of two-photon microscopy over conventional microscopy stem primarily from the inherent optical sectioning effect, relatively deep optical penetration, and flexible spectral accessibility.[3,4] More recently, two-photon imaging has entered the domain of endoscopy.[5,6] The use of two-photon fluorescence endoscopy could prove to be an important diagnostic tool, alleviating the need for surgical biopsy in both basic research and clinical pathology and producing spectra and images of tissue at the cellular level.

To achieve a compact and miniature microscope such as an endoscope, flexible fiber-optic components such as optical fibers, optical fiber couplers, and gradient-index (GRIN) rod lenses are usually integrated into the imaging system to replace complicated bulk optics.[7–9] A fiber-optic two-photon endoscope based on a single-mode optical fiber coupler, a GRIN rod lens, and a microprism has been constructed and exhibits an axial resolution of 3.2 μm.[6]

A GRIN rod lens has a parabolic-shaped refractive-index profile,[10] which gives rise to unique optical properties when compared with a normal microscope objective. The location of the focal point of a GRIN rod lens can be set by adjusting the magnitude of the gap between a source and the entrance face of the GRIN rod lens. Consequently, the numerical aperture (NA) of a GRIN rod lens is effectively changed as the gap between a source and a GRIN lens varies. Such an effective change in NA of a GRIN rod lens can affect the performance of fiber-optic two-photon fluorescence endoscopy[6] in three aspects. The first is imaging resolution that depends on the square of the effective NA,[11] the second is the illumination and collection efficiency that shows a complicated dependence on the effective NA, and the third is the two-photon fluorescence excitation strength that depends on the fourth power of the effective NA,[8,12] although the total two-photon excitation signal generated is not a function of the excitation NA.[12] Therefore a signal optimization of fiber-optic two-photon fluorescence endoscopy becomes possible and is necessary because the two-photon fluorescence signal from biological tissues is usually weak.

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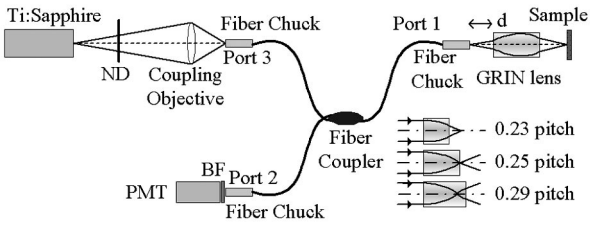

Fig. 1. Schematic diagram of the fiber-optic two-photon fluorescence microscope based on a fiber coupler and a GRIN rod lens.

In this paper we present an experimental investigation into the signal optimization of fiber-optic two-photon fluorescence microscopy. The effect of the gap between a fiber end and a GRIN rod lens on the effective NA of the GRIN rod lens is studied. In particular, the dependence of the two-photon fluorescence intensity on the magnitude of the gap is revealed for three GRIN rod lenses of typical pitch lengths. The optimization performance is demonstrated by the three-dimensional images of fluorescence beads in a fiber-optic two-photon fluorescence microscope.

2. Experimental Setup

A schematic diagram of the fiber-optic two-photon fluorescence microscope based on a fiber coupler and a GRIN rod lens is depicted in Fig. 1. An ultrashort-pulsed laser beam at a wavelength of 800 nm and with a pulse width of 80 fs at a repetition rate of 80 MHz generated by a turnkey Ti:sapphire laser (Spectra Physics, Mai Tai) is coupled through a microscope objective (Melles Griot, 20×/0.25 NA) into port 3 of the coupler. The fiber coupler used is a 50/50 three-port single-mode fiber coupler (Newport) designed for an operating wavelength of 785 nm. The coupler behaves as a low-pass filter in the visible wavelength range with a splitting ratio of between 99.6/0.4 and 99.7/0.3. The coupling efficiency from ports 3 to 1 is approximately 30% at a wavelength of 800 nm. The output beam from port 1 of the coupler crosses the gap and is focused by the GRIN rod lens. The fluorescence emitted from the sample is recollected by the GRIN rod lens and delivered by port 2 of the coupler into a photomultiplier tube masked with a 510 ± 20 nm bandpass filter (BF). A neutral density (ND) filter wheel placed before the coupling objective allows the variation of the input power.

Three GRIN rod lenses of pitches of 0.23, 0.25, and 0.29 to 830 nm (Newport) are chosen to confirm and compare the signal optimization condition in fiber-optic two-photon fluorescence microscopy. The difference in the pitch length implies that the focal position of the three GRIN rod lenses is at the surface of the lens, inside the lens, and outside the lens, respectively, if a collimated beam is used (see Fig. 1). The 1.8 mm diameter plano–plano GRIN lenses are designed for a wavelength of 830 nm. The 0.25 pitch GRIN lens has a NA of 0.6, while the NA of the 0.23 pitch and 0.29 pitch GRIN lenses is 0.46. The magnitude of the gap between the fiber coupler end and a GRIN lens d is controlled by a one-dimensional translation stage having a resolution of 10 μm. The maximum length of the gap is 5.5 mm to overfill the entrance face of the GRIN rod lens. The sample is a thin layer of AF-50 fluorescence dye, which has an average thickness of approximately 250 nm as measured by atomic-force microscopy.[8] The sample is driven by a scanning stage with a resolution of 0.1 μm and a 6 mm scanning range.

3. Determination of the Effective Numerical Aperture

To achieve the effective NA of a GRIN rod lens, the axial response of the system to a plane mirror is recorded by continuous scanning in the z direction. Measurement of the full width at half-maximum (FWHM) of the axial response curve allows for an analysis of the axial resolution of the system Δz, and then the effective NA of the GRIN rod lens, $\sin\alpha$, can be derived from

$$u = (8\pi/\lambda)\Delta z \sin^2(\alpha/2), \quad (1)$$

where u is an axial optical coordinate and λ is the wavelength of the illumination light.[11,13] By varying the gap length d, a set of axial response curves is obtained. The dependence of the FWHM of the axial response and the effective NA on the gap length for three given GRIN rod lenses is depicted in Fig. 2(a). It is shown that the FWHM of the axial response drops rapidly to approximately 13.6, 11, and 13 μm, respectively, as the gap length increases. The decrease in the FWHM indicates the improvement in axial resolution and therefore the enhancement of the effective NA of the GRIN lens. The measured effective NA reflects on the system NA that combines the excitation and emission NA. As expected, for all the GRIN rod lenses, the effective NA increases gradually and tends toward a limit (approximately 0.29, 0.31, and 0.28, respectively) when the gap increases until the output laser beam from the fiber coupler overfills the entrance face of the GRIN rod lens.

In addition, analysis of the peak intensity of the axial responses reveals a decrease in coupling efficiency of the system by approximately 71%, 81%, and 69%, respectively, as shown in Fig. 2(b). Such a signal reduction is caused by two physical processes. The first process is the coupling of illumination into a GRIN rod lens, which is confirmed by the transmitted power after the GRIN lens [Fig. 2(b)]. In all cases, the transmission efficiency for the input power exhibits a slight degradation of approximately 6.3%, 10%, and 6.3%, respectively, as a result of increasing the gap length d in the range between 0.25 and 5.5 mm. The second process is related to the mismatching between the fiber mode profile and the field distribution of the reflected signal on the end of the coupler. Once the fiber mode profile and the field distribution do not match each other, a significant decrease of collection efficiency will occur. It should be noted that the experimental results of the coupling efficiency in Fig.

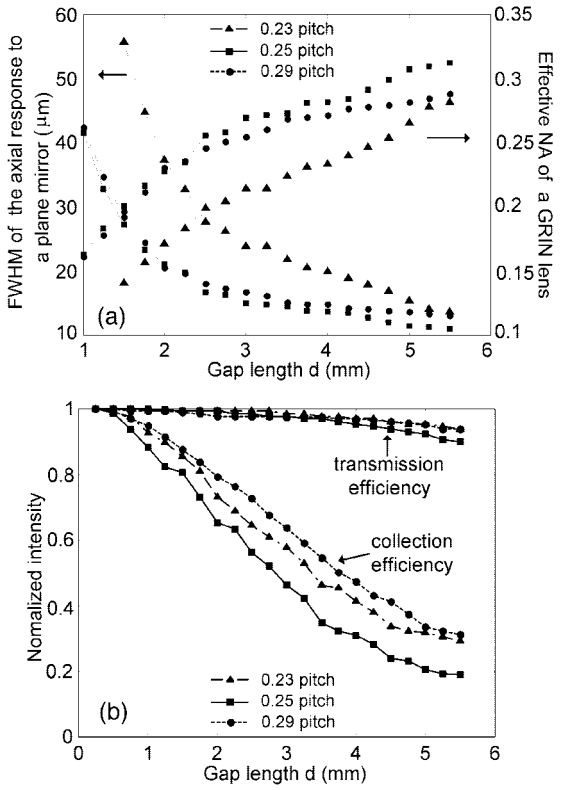

Fig. 2. One-photon reflection confocal axial response. (a) FWHM of the axial response to a plane mirror and effective NA of the GRIN rod lens as a function of gap d. (b) Transmission efficiency of the GRIN lens and peak intensity of the axial response to a plane mirror as a function of gap d.

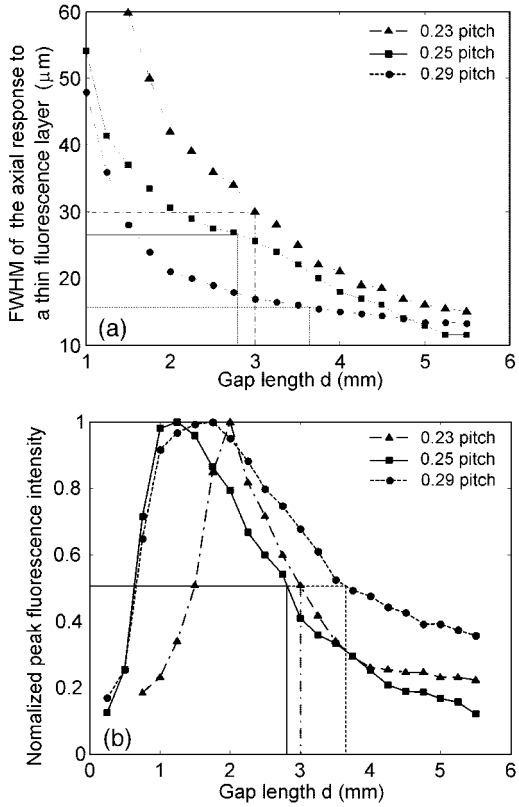

Fig. 3. Two-photon fluorescence axial response. (a) FWHM of the axial response to a fluorescence sheet as a function of gap d. (b) Peak intensity of the axial response to a fluorescence sheet as a function of gap d.

2(b) agree with the theoretical calculation of the signal level in reflection-mode fiber-optic confocal scanning microscopy, in the sense that the maximum reflected signal is achieved when the distance between the fiber and the GRIN lens is small.[13]

4. Optimization of a Two-Photon Fluorescence Signal

To study the two-photon fluorescence signal level in a fiber-optic two-photon microscope, a set of axial responses to the AF-50 fluorescence sheet is obtained. The FWHM of the axial response as a function of the gap length for the three GRIN rod lenses is plotted in Fig. 3(a), where the feature of the improvement in axial resolution is observed. We note that the axial resolution of two-photon imaging is 15, 11.6, and 13.3 μm for 0.23, 0.25, and 0.29 pitch GRIN rod lenses, respectively, when the gap length is 5.5 mm.

Figure 3(b) shows the peak two-photon fluorescence intensity of the axial response as a function of gap length for the three GRIN lenses. It is clear that the maximum fluorescence intensity is collected by the fiber coupler over the gap range between 0.25 and 5.5 mm in all cases. Further observation from Fig. 3(b) reveals that the maximum fluorescence signal appears at gap lengths of approximately 2, 1.25, and 1.75 mm for the three GRIN lenses. The appearance of the maximum two-photon signal results from two competing physical processes. On the one hand, increasing the effective NA leads to the quick enhancement of the two-photon fluorescence signal because the two-photon excitation probability is proportional to the fourth power of the effective NA.[12] On the other hand, as suggested in Fig. 2(b), the illumination and collection efficiency of the imaging system drops as the effective NA increases. Therefore, although the emitted two-photon fluorescence signal increases with the increase of the gap length, the two-photon fluorescence signal coupled backward by the fiber eventually reduces, as shown in Fig. 3(b).

The combination of Figs. 3(a) and 3(b) implies that there is a trade-off between the two-photon fluorescence axial resolution of the system and the two-photon fluorescence signal level that is related to the parameters of the GRIN lens and the optical wavelength. At the maximum signal level, the corresponding axial resolution is 42.0, 38.5, and 24.0 μm, respectively, for 0.23, 0.25, and 0.29 pitch GRIN rod lenses. Consider the gap length at which the two-photon fluorescence signal level drops to 50% of the maximum [Fig. 3(b)]. The axial resolution corresponding to the three GRIN rod lenses is approximately 30.0, 26.0, and 15.7 μm [Fig. 3(a)]. Table 1 summarizes the effective NA, optimized axial resolution, and axial resolution at maximum fluorescence intensity, etc., which would be considered in applications of two-photon fluorescence endoscopy.

Table 1. Characterization of the GRIN Rod Lens in a Fiber-Optic Two-Photon Fluorescence Microscope

GRIN Lens Pitch at 830 nm	Maximum NA	Effective Confocal NA	Optimized Confocal Δz (μm)	Optimized TPF Δz (μm)	TPF Δz at Half-Maximum Intensity (μm)	TPF Δz at Maximum Intensity (μm)	d at Maximum Intensity (mm)
0.23	0.46	0.29	13.6	15	30	42	2
0.25	0.6	0.31	11	11.6[a]	26	38.5[a]	1.25
0.29	0.46	0.28	13	13.3	15.7	24	1.75

[a]These axial resolution values are shown in Fig. 4 with 10 μm beads at 1/3 the axial focus spacing. Δz is the axial resolution, and TPF represents two-photon fluorescence.

The appearance of the maximum signal level when the gap length increases can be understood from the optical transfer function analysis.[14] According to this method,[11,14] the signal level of a thin fluorescence sheet is proportional to the value of the two-dimensional in-focus optical transfer function $C_2(l)$ at $l = 0$, where l is the transverse spatial frequency of an object.[11] In the case of the fiber-optic two-photon fluorescence microscope that uses a single-mode fiber coupler and a GRIN rod lens, the signal level can be expressed, if the input power from the Gaussian fiber profile and the power loss by the GRIN rod lens are considered, as

$$\eta_2 = \frac{\beta A^4 C_2(l=0)}{1-\exp(-A)}, \tag{2}$$

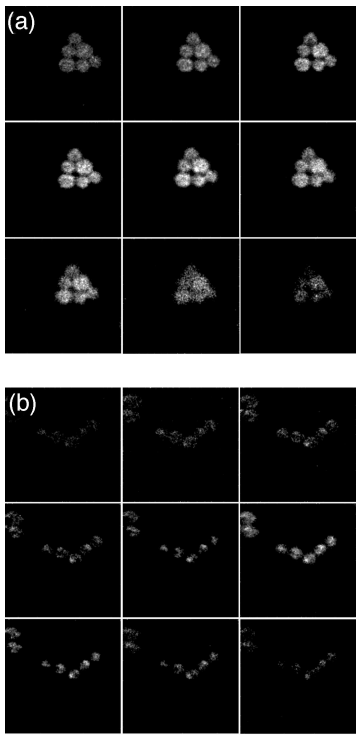

Fig. 4. Series of x–y images of 10 μm fluorescence polymer microspheres acquired with a 0.25 pitch GRIN lens by (a) setting d = 1.25 mm with a slice spacing of 15 μm and (b) setting d = 5.5 mm with a slice spacing of 5 μm. The axial resolution for these two gap lengths are shown in Table 1. Each slice size is 100 μm × 100 μm. The average power on the sample is 10 mW.

where β is a factor of normalization and $A = [2\pi a r_0/(\lambda d)]^2$. Here a and r_0 are the radius of the GRIN rod lens and the fiber mode profile, respectively, λ is the illumination wavelength, and d is the gap length. As was shown in an earlier study,[14] $C_2(l = 0)$ decreases monotonically as A increases. As a result of the balance between $A^4/[1 - \exp(-A)]$ and $C_2(l = 0)$, the signal level given by Eq. (2) leads to a maximum peak, qualitatively confirming the behavior of Fig. 3(b).

To show the optimum imaging performance of the fiber-optic two-photon fluorescence microscope, the two image sets of 10 μm fluorescence polymer microspheres shown in Fig. 4 are obtained with the 0.25 pitch GRIN rod lens when the gap between the fiber coupler end and the GRIN rod lens is 1.25 and 5.5 mm, respectively. The two image sets are recorded with a lateral size of 100 μm and a slice spacing of 15 and 5 μm, respectively. It is clearly observable that the two-photon fluorescence images have strong intensity but poor resolution with the gap where the optimization of the signal level is achieved [Fig. 4(a)]. On the other hand, the optical sectioning ability of the system is optimized with a poor signal level when the gap distance is at maximum [Fig. 4(b)].

5. Conclusion

We have demonstrated that the fluorescence signal level and axial resolution are functions of the gap between the fiber coupler end and the GRIN rod lens for a fiber-optic two-photon fluorescence microscope. The collected two-photon fluorescence intensity exhibits a maximum value as the gap between the fiber coupler end and the GRIN rod lens enlarges, i.e., as the effective NA of a GRIN lens increases. The optimal two-photon fluorescence signal level appears at a gap length of approximately 2, 1.25, and 1.75 mm for a GRIN rod lens of pitches 0.23, 0.25, and 0.29. The performance of the fiber-optic two-photon microscope is demonstrated by the three-dimensional images of fluorescence microspheres. These results elucidate choices in the signal-to-resolution trade-off in the application of fiber-optic two-photon fluorescence endoscopy to achieve visualization of biological tissues.

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