

Two-photon fluorescence scanning near-field microscopy based on a focused evanescent field under total internal reflection

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We present two-photon fluorescence near-field microscopy based on an evanescent field focus produced by a ring beam under total internal reflection. The evanescent field produced by this method is focused by a high-numerical-aperture objective, producing a tightly confined volume that can effectively induce two-photon excitation. The imaging system is characterized by the two-photon-excited images of the nanocrystals, which show that the focused evanescent field is split into two lobes because of the enhancement of the longitudinal polarization component at the focus. This feature is confirmed by the theoretical prediction. Unlike other two-photon near-field probes, this method does not have the heating effect and requires no control mechanism of the distance between a sample and the probe. © 2003 Optical Society of America
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Far-field two-photon microscopy is a well-established technique in fluorescence microscopy.¹ The advantages of the two-photon technique are a significant reduction in the focal excitation volume because of the quadratic excitation dependence on intensity, less photodamage to the sample, and the ability to acquire a full fluorescence spectrum because its excitation is far from the emission wavelengths. Recently, the two-photon excitation technique was extended to near-field fluorescence microscopy,²⁻⁵ in which the reduction in excitation volume caused by two-photon excitation was shown to further improve its resolution and contrast.

To date, either near-field probes that incorporate subwavelength aperture fiber tips with⁴ or without metal coating^{2,3} or apertureless metallic tips⁵ have been employed for two-photon excitation. The use of an uncoated fiber tip with a tip apex of 200 nm has shown a resolution of ~175 nm,² whereas the use of a metallic tip greatly enhances the field near the tip, producing a resolution of ~40 nm with a tip apex of 10 nm.⁵ Despite their improvement in optical resolution, these methods are difficult to use because a near-field tip must be kept at a close distance from the sample. This procedure can easily result in damage to the probe tip. In addition, the absorption of light in the metallic coating of a fiber tip or a metallic tip causes significant heating of the sample and poses a problem for biological applications. Furthermore, it is extremely difficult to coat metal smoothly onto fiber tips on the nanometer scale.

To overcome these problems, one may employ a focused evanescent field as a two-photon near-field probe. In a recent development a new type of near-field imaging microscopy called scanning total internal reflection fluorescence microscopy (STIRFM)

was proposed⁶ in which a focused evanescent field is employed as a sensing probe. The use of a ring beam and a high-numerical-aperture objective produces a tight confinement of the evanescent field, and the enhanced evanescent focal spot makes it possible to excite a nonlinear effect such as two-photon absorption. In this Letter we demonstrate two-photon-excited near-field fluorescence imaging with STIRFM. The new instrument is characterized by the image of CdSe quantum dot nanocrystals (NCs). This new technique is advantageous over other near-field probe techniques because of its highly reproducible nature, no heating of the sample or probe-sample distance control, and the ability to operate in a far-field microscope.

The experimental setup is shown in Fig. 1. We used a Ti:sapphire ultrashort-pulsed laser (Spectra-Physics Tsunami) operating at a wavelength of 800 nm. The linearly polarized laser beam was expanded and focused at the glass-air interface by an objective (numerical aperture, 1.65; Olympus). A central obstruction disk was inserted just before the reflection at the dichroic beam splitter, producing a ring-beam illumination. The diameter of the obstruction disk was chosen such that all the beams with a convergence angle smaller than the critical angle of incidence ($\theta_c = 35^\circ$) could be obstructed. The incident in-plane polarization could be rotated by insertion of a quarter-wave plate and a Glan-Thompson polarizer in the illumination path, with a polarization extinction ratio of 100,000:1. The two-photon-excited fluorescence from the focus was then collected by the same objective and refocused at a photomultiplier tube (PMT) (Oriel PMT Model 70680). A 200- μm -diameter pinhole was placed in front of the PMT to reduce the background scattered light. The sample was scanned in the x and y directions by a scanning stage (Physik

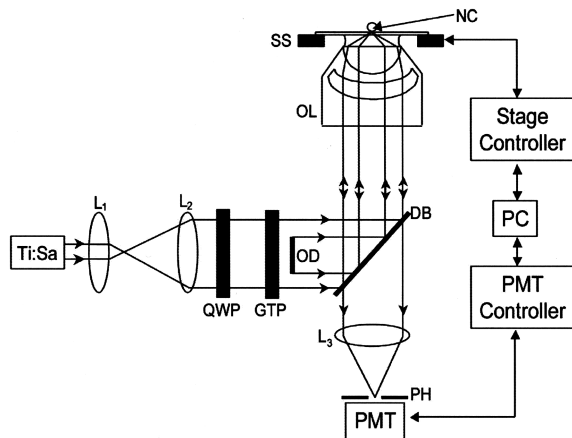


Fig. 1. Experimental setup: QWP, quarter-wave plate; GTP, Glan–Thompson polarizer; OD, obstruction disk; SS, scanning stage; DB, dichroic beam splitter; NC, nanocrystals; OL, objective lens (numerical aperture of 1.65); Ti:Sa, Ti:sapphire laser; L_1 – L_3 , lenses; PMT, photomultiplier tube; PH, pinhole; PC, personal computer.

Instrumente Model P-517.3CL) to build up a two-dimensional fluorescence image. A special coverslip glass and immersion oil for the 1.65-numerical-aperture objective had a refractive index of 1.78. The high refractive index of the immersion oil and the coverslip glass increased the portion of the objective exit pupil in producing an evanescent field.

To characterize the two-photon evanescent focus, we used CdSe quantum dot NCs as an imaging sample. The semiconductor NCs have several advantages in characterizing the focal spot of the current imaging system. First, because of their small size (<5 nm) compared with the focal spot (~ 200 nm), they can act as a fluorescent point source in image convolution. Second, their relatively spherical shape makes them insensitive to the excitation polarization selectivity,⁷ making it possible to probe the total field and not just a specific polarization component at the focus. CdSe quantum dot NCs capped with trioctylphosphine oxide and trioctylphosphine were prepared with the method described in Ref. 7. The NCs had a mean diameter of 3 nm, and the peak fluorescence wavelength was at 530 nm. The NCs were diluted in chloroform, and a droplet of the solution was dried onto a cleaned special coverslip glass. The average density of the quantum dots on the coverslip was 1 quantum dot/ μm^2 .

Figure 2 shows typical two-photon fluorescence images of the NCs. The dependence of the NCs' fluorescence on the laser input power [Fig. 2(a)] is approximately quadratic (gradient of 2.0039 in the log–log plot), confirming two-photon absorption by the NCs. The spot shapes in Fig. 2(b) show two distinctive lobes separated by approximately 200 nm, oriented along the direction of the incident polarization. The individual lobe of the split focus has a FWHM of approximately 150 nm. This feature indicates the excitation of NCs by enhanced longitudinal-polarization components at the focus, caused by the strong depolarization effect of a high-numerical-aperture objective.⁸ The detailed theoretical evanescent focal shape is discussed below.

Slight asymmetry of two-peak spots is believed to be caused by both the imperfect plane-wave front of the input beam and the overlap of the two lobes of proximal NCs. The two-peak spots may not necessarily be the image of a single NC, since the size of the focal spot is much larger than the size of an individual NC. A single NC may be identified by monitoring of the fluorescence intermittency that is caused by Auger ionization of the NCs.⁹ However, in our study the fluorescence intermittency was not observed during the imaging because of the fast imaging speed ($80 \mu\text{m/s}$).

To confirm that the split focus is not caused by artifacts, we rotated the incident in-plane polarization direction with the Glan–Thompson polarizer and observed the direction of the focus splitting. As shown in Figs. 2(b)–2(e), the direction of the splitting is also rotated according to the incident polarization direction. The intensity dependence on the excitation polarization direction was negligible in our experiment, although such a dependence of a single NC was predicted because of intrinsic transition dipole orientations of individual states¹⁰ or the ellipticity of the NC shape.¹¹ Because of the broad pulse spectrum used in our experiment, it is likely that several overlapping electronic states within a single NC are excited simultaneously, effectively reducing the excitation polarization dependence.

The theoretical two-photon fluorescence profile along the direction of the incidence polarization is calculated with the vectorial Debye theory¹² in the presence of an interface. According to the theory, this split focus profile is produced by the complicated mixture of the transverse-field component E_x and the enhanced longitudinal-field component E_z .⁸ In the case of the interface used in our experiment the strength of the longitudinal field reaches 80% of that of the transverse field when the normalized obstruction size ϵ is approximately 0.6 (see the inset in Fig. 3). Figure 3 shows the relative two-photon fluorescence intensity profiles of the longitudinal (I_z), transverse

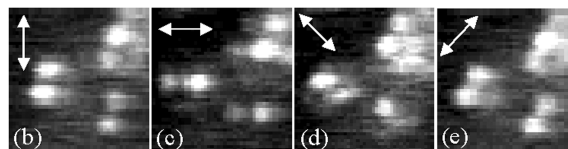
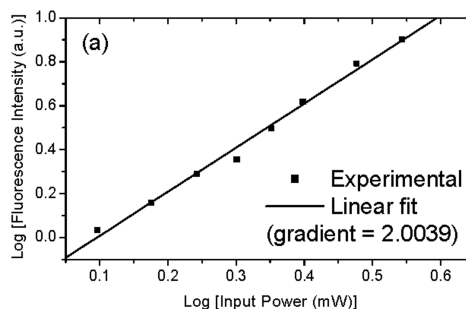


Fig. 2. (a) Dependence of the two-photon fluorescence intensity on the evanescent intensity. (b)–(e) Direction of the focus splitting with respect to the incident polarization ($1.4 \mu\text{m} \times 1.4 \mu\text{m}$) under an illumination of 0.2 MW/cm^2 . The arrows indicate the direction of the incident polarization.

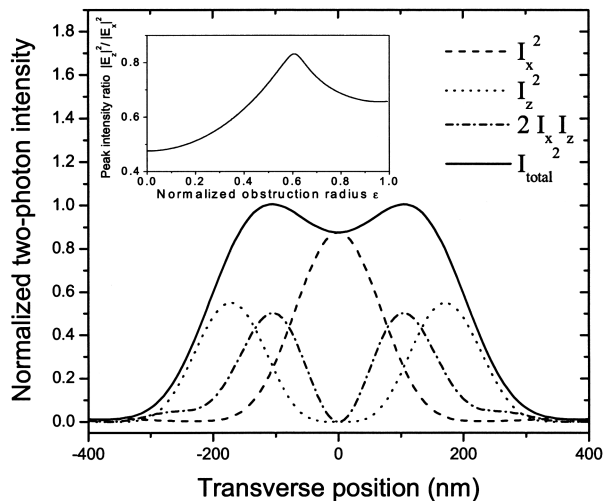


Fig. 3. Normalized two-photon theoretical fluorescence profile and contributions from individual field components along the direction of the incident polarization. Inset, plot of the peak intensity ratio $|E_z|^2/|E_x|^2$ as a function of the normalized obstruction radius ϵ at the interface between the coverslip glass ($n = 1.78$) and air (numerical aperture of 1.65).

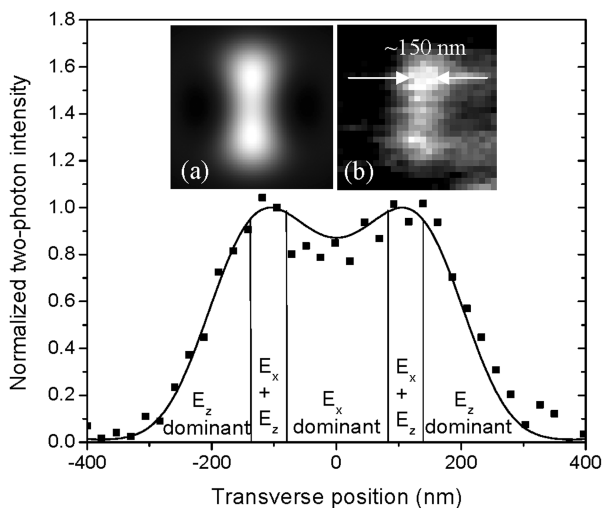


Fig. 4. Comparison between theory (solid curve) and an experimental split-focus profile (squares) along the incident polarization direction and mapping of the excitation field components. The experimental data was averaged five times. Insets (a) and (b), close-up views of theoretical and experimental images ($600 \text{ nm} \times 600 \text{ nm}$), respectively.

(I_x), and total field components ($I_x + I_z$). It should be pointed out that the strength of the I_y component is generally 2 orders of magnitude weaker than the I_x and I_z components. Note that there is another contribution ($2I_x I_z$) to the total field from the two-photon absorption [i.e., $I_{\text{total}}^2 = (I_x + I_z)^2 = I_x^2 + I_z^2 + 2I_x I_z$]. As a result of this nonlinear dependence, the dip depth of the two-photon fluorescence profile reaches 20% of the peak intensity, making the split focus discernible.

According to the relative field strength of each component, the split focus can be mapped into three

regions, as indicated in Fig. 4. The distinction among regions is made so that the contribution from one component of the three components (i.e., I_x^2 , I_z^2 , and $2I_x I_z$) exceeds 45% of the total intensity. The transverse-field component dominates in the region near the optical axis (dip between the two peaks), the transverse and longitudinal fields both exist in the region near two peaks, and the longitudinal field dominates in the region further from the two peaks. A comparison of the experimental and theoretical profiles is also given in Fig. 4, showing excellent agreement. This result confirms that the excitation cross section of CdSe NCs is isotropic in all three directions, as expected.⁷

In conclusion, it has been demonstrated that the focused evanescent field in STIRFM can be used for two-photon excitation. A two-photon fluorescence near-field microscope has been well characterized by the use of CdSe NCs as an effective point object. An important feature of this type of nonlinear near-field microscopy is its enhanced longitudinal-field component. This feature, together with no heating and no distance control properties, may prove advantageous when this technique is applied to single molecular imaging. In the future the resolution of the current technique can be further improved by removing one of the two lobes present in the split focus with the help of lens apodization.

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