Hyper-numerical aperture (NA = 2.8) microscope using $\lambda = 1.56 \, \mu m$ femtosecond source for multi-photon imaging

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Abstract: A new microscope is discussed, where the scanning illumination has a numerical aperture of 2.8 with $\lambda = 1.56 \, \mu m$ femtosecond fiber laser. Samples are placed or grown on a silicon substrate. Multi-photon emission is imaged in transmission on a cooled CCD. Two-photon and three-photon effects are observed from the silicon/water interface and gold nanoparticles. Images of cells, reference spheres and gold nanoparticles illustrate imaging properties of the microscope. Spectral characteristics of individual particles are achieved with a blazed transmission grating. Emission properties of differently sized gold nanoparticles are studied in detail, which indicate that their emission is a two-photon effect due continuum generation. Interestingly, spectral shape and emission power are similar for 20nm, 40nm and 60nm diameter gold nanoparticles for the cases studied.

OCIS codes: (180.4243) Near-field microscopy; (180.4315) Nonlinear microscopy.

References and links

1. P. T. C. So, C. Y. Dong, B. R. Masters, and K. M. Berland, “Two-photon excitation fluorescence microscopy,” Annu. Rev. Biomed. Eng. 2(1), 399–429 (2000).
2. D. Kobat, M. E. Durst, N. Nishimura, A. W. Wong, C. B. Schaffer, and C. Xu, “Deep tissue multiphoton microscopy using longer wavelength excitation,” Opt. Express 17(16), 13354–13364 (2009).
3. J. A. Squier, M. Muller, G. J. Brakenhoff, and K. R. Wilson, “Third harmonic generation microscopy,” Opt. Express 3(9), 315–324 (1998).
4. A. C. Millard, P. W. Wiseman, D. N. Fittinghoff, K. R. Wilson, J. A. Squier, and M. Müller, “Third-harmonic generation microscopy by use of a compact, femtosecond fiber laser source,” Appl. Opt. 38(36), 7393–7397 (1999).
5. G. Rago, B. Bauer, F. Svobdberg, L. Gunnarsson, M. B. Ericson, M. Bonn, and A. Encejd, “Uptake of gold nanoparticles in healthy and tumor cells visualized by nonlinear optical microscopy,” J. Phys. Chem. B 115(17), 5008–5016 (2011).
6. X. Huang, W. Qian, I. H. El-Sayed, and M. A. El-Sayed, “The potential use of the enhanced nonlinear properties of gold nanospheres in photothermal cancer therapy,” Lasers Surg. Med. 39(9), 747–753 (2007).
7. S. Hell, G. Reiner, C. Cremer, and E. H. K. Stelzer, “Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index,” J. Microsc. 169(3), 391–405 (1993).
8. A. Rohrbach and E. H. K. Stelzer, “Trapping forces, force constants, and potential depths for dielectric spheres in the presence of spherical aberrations,” Appl. Opt. 41(13), 2494–2507 (2002).
9. J. Zhang, Y. Kim, Y. S. Kim, R. Valencia, T. D. Milster, and D. Dozer, “High resolution semiconductor inspection by using solid immersion lenses,” Jap J. Appl. Phys. 48, 03A043 (2009).
10. J. Zhang, Y. S. Kim, S. H. Yang, and T. D. Milster, “Imaging artifacts in hyper-NA vector imaging,” J. Opt. Soc. Am. A 27(10), 2272–2284 (2010).
11. S. M. Mansfield and G. S. Kino, “Solid immersion microscope,” Appl. Phys. Lett. 57(24), 2615–2616 (1990).
12. S. H. Yang, T. D. Milster, J. Zhang, and T. Chen, “Characteristics of evanescent polarization imaging,” J. Mod. Opt. 57(9), 783–797 (2010).
13. T. D. Milster, J. S. Jo, and K. Hirota, “Roles of propagating and evanescent waves in solid immersion lens systems,” Appl. Opt. 38(23), 5046–5057 (1999).
14. A. L. Mattheyes, S. M. Simon, and J. Z. Rapprport, “Imaging with total internal reflection fluorescence microscopy for the cell biologist,” J. Cell Sci. 123(21), 3621–3628 (2010).
15. K. Kieu, R. J. Jones, and N. Peyghambarian, “Generation of few-cycle pulses from an amplified carbon nanotube mode-locked fiber laser system,” IEEE Photon. Technol. Lett. 22(10), 1521–1523 (2010).
1. Introduction

The application of multi-photon excitation (MPE) to fluorescence microscopy has become a powerful tool for studying biological function in live tissue and offers many advantages over conventional imaging techniques, such as submicron resolution, reduced photodamage and good depth penetration [1]. MPE also offers high sensitivity imaging by eliminating contamination of the fluorescence signal by excitation light. Recently, interest in MPE has extended to infrared excitation, due to better depth penetration [2]. Although two-photon excitation fluorescence (2PEF) is usually the primary signal source in multi-photon microscopy, three-photon excitation fluorescence (3PEF), second-harmonic generation (SHG) and third harmonic generation (THG) can also be used for imaging [3,4]. In addition, MPE properties of gold nanoparticles (GNPs) make them attractive for imaging [5] and therapy [6], because they do not bleach and are relatively nontoxic. In this paper, we discuss development of a new microscope that is designed for MPE at the 1.56 μm excitation wavelength. Unique aspects of the microscope include a hyper-numerical-aperture (hyper-NA) optical system with cells grown on a silicon substrate, which provide scanning-spot resolution well below conventional scanning microscopes at this excitation wavelength and a mirror effect that collects twice the emitted light. An insertable blazed diffraction grating allows direct optical spectrum analysis of emission points in the object field, which show interesting behavior with respect to individual GNPs.

As is well known, increasing numerical aperture often leads to better resolution. However, the imaging system must be corrected for aberrations. If not, one often finds that a lower-NA system produces better results than a higher NA system [7,8]. Like oil-based high NA systems, hyper-NA systems (which have NA > 1.5), utilize high-refractive index material in proximity to the object being imaged. In previous work, the authors applied silicon material (n = 3.5 at λ = 1.2 μm) to generate an imaging system with NA = 2.45 that was used to examine computer chips [9], and off-axis polarized monopole illumination to hyper-NA system [10]. A similar system is described in this work, where the substrate onto which cells are grown and test objects placed is made of silicon.

A critical element of the optical system is a silicon solid immersion lens (SIL) in the illumination path. The SIL, in combination with the silicon substrate, forms an object-centric hemisphere. The backing objective lens (Olympus LPLFL100XIR NA = 0.8) focuses through the SIL and onto the sample side of the substrate to realize NA = 2.8 with diffraction-limited aberrations over a 30μm object field at the infrared (IR) wavelength of 1.56μm.

The SIL, which was originally introduced by Mansfield and Kino [11], contains a mixture of evanescent and propagating waves at the sample surface [12,13]. To realize full resolution potential of a SIL, the object must be within the evanescent decay portion of the illuminating field. This restriction is similar to the advantage of total internal reflection fluorescence microscopy (TIRF), where only structures within about 100nm of the substrate surface are imaged [14]. However, the SIL focus beam contains a spectrum of plane-wave angles that have different decay lengths, as well as a significant amount of propagating energy.

The experimental light source is a convenient mode-locked fiber-based femtosecond pulsed laser (KPhotonic model CNT-1150-TK-A) that uses a saturable absorber based on a fiber taper coated with single-walled carbon nanotubes. The center wavelength is 1.56 μm with a bandwidth of 30nm, pulse width of 150 femtoseconds at the laser output, repetition...
rate of 40 MHz, and average output power of 60 mW [15]. This pulsed laser has been used with NA ~0.5 to generate second- and third-harmonic generation (SHG, THG), and two- and three-photon excited fluorescence (2PEF, 3PEF) from biological samples [16].

In this paper, construction of the microscope and the sample is described in detail in Section 2. Section 3 discusses results of several imaging experiments with reference spheres, cell and GNPs. Different imaging modes are illustrated, including using the blazed diffraction grating for spectral analysis of the MPE from GNPs. Emission characteristics of differently sized GNPs are studied in detail. Section 4 lists primary conclusions from this work.

2. Sample and microscope construction

The sample holder is a unique aspect of this microscope. As shown in Fig. 1, the sample holder substrate is made from a 400μm thick silicon wafer that is optically polished on both sides. A 2.6mm thick and 3.0mm radius object-centric hemispherical SIL is coupled directly to the bottom of the substrate. The substrate surfaces and the flat surface of the SIL are polished to about 1nm rms roughness and 3nm rms roughness, respectively, and the two are coupled with a small amount of high-index immersion oil. With the 0.8 NA backing objective lens, effective NA at the sample surface is $NA = 3.5 \times 0.8 = 2.8$. Full-width-at-half-maximum (FWHM) spot size is about 400nm for the 1.56μm wavelength and 230nm for the THG wavelength of 0.52μm. Experimentally, it was determined that cells can be grown easily on the silicon substrate.

![Sample geometry diagram](image)

Fig. 1. Sample geometry using silicon sample substrate and object-centric silicon solid-immersion lens (SIL). The excitation wavelength at 1.56μm is focused through the SIL and onto the sample surface through a 0.8NA backing objective lens. Objects are placed/grown on the top side of the silicon substrate. The laser is scanned across the sample surface to produce an image. Excitation full-width-at-half-maximum values from the actinic 1.56μm laser and $P'$ at 0.52μm are 400nm and 230nm, respectively.

Actinic laser wavelength epi reflection from the sample surface is detected with a multispectral camera (TriWave EC701) for alignment, and MPE produced in the sample is collected in transmission. Samples are prepared in standard nutrient solutions or DI water with an Invitrogen 0.12mm deep adhesive spacer attached to the substrate. Before scanning, a 0.5mm thick fused silica coverslip is attached to the top side of the adhesive spacer. The non-standard coverslip is used to facilitate higher-NA collection in future versions of the microscope. In the work reported here, collection is accomplished through one of several Olympus objective lenses, including an LMPlanFL (100X 0.8NA), LWD MSPlan100LCD (100X 0.8NA) with adjustable coverplate thickness compensating ring, and a UPlanFl (10X 0.3NA). The 100LCD adjustment is optimized during collection to provide best image resolution. The LMPlanFL 100X can also be used with a fused silica SIL to effect NA = 1.2 for collection. The image is detected on a cooled CCD (SBIG model ST-402ME), and distinguished between 2PEF, SHG, 3PEF, and THG spectra through the application of a
blazed transmission grating. Collectively, this paper refers to these effects as MPE. The imaging camera is unresponsive to the actinic laser wavelength.

Although extremely small spot size and high energy density is realized at the sample surface, the focus cone contains approximately 87% evanescent energy at the laser wavelength that eventually decays through the sample volume or is converted to propagating energy. Figure 2 shows detailed vector calculations of spot profiles and relative power versus depth in a uniform water sample at the laser wavelength. Linear x-polarization is assumed in the entrance pupil of the illuminating objective lens. Due to vector focusing effects, the x-direction spot size is slightly larger than in the y direction. 2P and SHG excitation go as $I^2$, where 3P and THG excitation go as $I^3$. Excitations in both cases decay quickly from the substrate surface, with one order of magnitude decreases for $I^2$ and $I^3$ at distances of 170nm and 100nm, respectively, away from the interface. In addition to $I^2$ and $I^3$ effects in the sample, it is well known that there is also a significant amount of THG generated at the liquid/substrate interface near focus [17].

If an effective focal depth of 200nm and spot diameter of 400nm is used in the calculations, illumination focus volume for the 2.8 NA solid immersion lens at 1.56μm is 0.025 femtoliters. With an average power of 60mW, transmission efficiency of 20% to the sample, pulse width of 150 femoseconds and repetition rate of 50 MHz, 0.24 nJ/pulse and 1.6 kW peak power is generated at the sample. The power per unit area is $1.27 \times 10^{12}$ W/cm² at focus.

Geometry of the multi-photon microscope is shown in Fig. 3(a). A flip mirror selects either a HeNe alignment laser or the 1.56μm fs laser. Laser reflection from G1 (x-scan galvanometer) is imaged onto G2 (y-scan galvanometer) with Relay 1. Relay 2 images the reflection from G2 into the entrance pupil of the illumination objective lens. The full scan range with 2.8 NA is about 30μm in x and y. An infrared LED (IR LED) at $\lambda = 1.2$μm illuminates the sample from below for epi reflection during alignment. IR images are collected on the IR Camera.
Fig. 3. Microscope geometry. (a) Basic vertical column construction with laser sources and galvanometer mirrors horizontal on an optical table: L1: 0.6328μm HeNe alignment laser, L2: 1.56μm fs laser, G1 and G2: galvanometer mirrors, Relay 1 and Relay 2 are afocal relays. A blazed transmission grating is inserted in the collection path to view MPE spectra. (b) Photograph of the flexure housings for precisely locating SILs on the sample relative to the objective lenses.

After MPE is generated in the sample, it is collected by one of several objective lenses, ranging from 0.3NA to 1.2NA. The visible LED provides epi-illumination with selectable center wavelengths of 623nm, 525nm or 465nm. Visible-light LED epi-illumination is useful with concurrent MPE detection to correlate sample structure to MPE signals. In addition, a shorter wavelength source could be used to excite fluorophores in biological samples. A blazed grating with finesse = 55 can be inserted in the collection path in order to observe spectra of individual nanoparticles. As shown in Fig. 3(b), backing objectives for both illumination and collection SILs are mounted in z-axis flexure housings that precisely locate the SILs with respect to optical axes of the objectives. As shown, both SILs are used. However, in these experiments the collection SIL is not used, so neither is the collection flexure housing.

3. Results and discussion

The different imaging modes of the microscope are useful to locate and analyze images. For example, Fig. 4 shows images of a substrate coated with 4μm diameter fused silica spheres, 250nm diameter GNPs and sparsely distributed 60nm GNPs. In Fig. 4(a), 625nm epi LED illumination is combined with 2.8NA scanning of the 1.56μm wavelength fs laser. The scan area is easily observed, due to THG from the silicon/water interface. Figure 4(b) show the same field, but with the epi illumination turned off. Although a 250nm diameter GNP is clearly visible in the scan range, it does not produce measurable MPE. However, MPE from a single 60nm diameter GNP is visible on the left edge of the scan range. A slight nonuniformity of the THG at the top and bottom of the scan range is due to an artifact in the scan electronics.

Figure 5 shows images of yeast cells combined with 4μm diameter fused silica spheres, 250nm diameter GNPs and 60nm diameter GNPs. Figure 5(a) shows the red epi illumination image, where yeast, 4μm fused silica spheres and 250nm GNPs are clearly visible. Like in Fig. 4, the 60nm GNPs are not resolved in the epi image. In Fig. 5(b), the epi illumination is
turned off, and 2.8NA scanning with the 1.56μm fs laser is turned on. THG from the silicon/water interface clearly shows the scan area, which is slightly curved on the left side due to vignetting in the scan optics. MPE is also clearly visible from the 60nm diameter GPNs. Note that the yeast cells are alive and active. They typically move between image frames. In all four images, image frames cover the same sample area. In Fig. 5(c), a green blocking filter is inserted into the collection optics, so only wavelengths longer than 540nm pass through to the camera. THG from the silicon/water interface is removed from the image. In Fig. 5(d), the blazed grating is also inserted into the collection optics, and GNP spectra are recorded as streaks across the image. Note that the grating disperses light in the image according to wavelength, where longer wavelengths are dispersed further to the right. A broad spectrum two-photon emission (2PE) is observed at most of the 60nm GNP sites. However, an occasional SHG emission is observed, which is characterized by an emission peak near 780nm. Spectra are calibrated by using a vertical line scan (not shown) and measuring separation of the THG diffraction orders. Because the brazed grating is efficient in the visible spectrum, most of the transmitted energy is directed into the +1 diffracted order. Although THG from the cells are not observed due to the strong silicon/water interface THG, it may be possible to filter this interface THG with a simple spatial blocking filter in the collection pupil, as suggested by Lippitz et al [18].

![Image](https://example.com/image1.png)

**Fig. 4.** Images of 4μm diameter fused silica spheres, 250nm diameter GPNs and sparsely distributed 60nm diameter GPNs. (a) 625nm red epi illumination concurrent with 2.8NA scanning 1.56μm wavelength fs laser; and (b) 2.8NA scanning 1.56μm wavelength fs laser only.

Emission properties of the GPNs are interesting and are examined in more detail. Several substrates are prepared with GPNs of 20nm, 40nm and 60nm diameters. A scanning electron microscope was used to verify that the particles were sparsely dispersed on the substrate and not clumped. Images were taken of individual GPNs with the blazed grating installed and the laser beam directly illuminating each particle. That is, scanning was stopped during image acquisition. Collection NA = 0.3 with the 10X objective lens.

Figure 6(a) shows spectra derived from three 60nm diameter spherical GPNs obtained with a 0.5 sec integration time. Also included are spectra from three locations of the laser beam on the silicon substrate without any GNP, which is labeled as background. An image is processed by windowing a portion of the image corresponding to the width of the emission in the direction perpendicular to the dispersion of the grating and summing 16-bit image pixel values in this direction to create a one-dimensional signal curve. Signal curves are normalized to their maximum value. THG from the substrate is significant between about 500nm to 545nm. The maximum continuum emission at about 650nm is approximately 6% of the THG, although the GPNs differ slightly in their response. A detailed view of the continuum emission is shown in Fig. 6(b), where a software notch filter is applied to zero signals in the range where THG is significant. Curves are renormalized to their maximum values after the notch filter is applied. The GPNs exhibit nearly the same spectral response. Comparatively,
the background signal from the substrate-only signal is only about 5% of the maximum continuum signal at 650nm.

Fig. 5. Images of yeast cells, 4μm diameter fused silica spheres, 250nm diameter GNPs and 60nm diameter GNPs. (a) 625nm red epi illumination; (b) 2.8NA scanning 1.56μm wavelength fs laser only; (c) Red epi illumination with a green blocking filter in the collection optics and 2.8NA scanning 1.56μm wavelength fs laser; and (d) 2.8NA scanning 1.56μm wavelength fs laser (no epi) with the blazed grating inserted in the collection path. Longer wavelengths are dispersed to the right. Most of the transmitted light is directed into the + 1 diffraction order.

Fig. 6. Spectra of three 60nm diameter GNPs on a silicon substrate: a) Signal curves normalized to their maximum values of the THG; and b) Signal curves renormalized after application of a software notch filter from 500nm to 545nm.
Fig. 7. Power analysis of 60nm diameter GNP emission. Two sections of the signal curve are integrated and displayed on the log-log plot. The first section that corresponds to THG from the silicon substrate, from 500nm to 545nm, clearly shows a $I^3$ response with slope $m = 2.8$. The second section that corresponds to continuum emission, from 400nm to 900nm and excluding the 500nm to 545nm notch filter, clearly displays a $I^2$ response with slope $m = 1.95$.

Figure 7 shows MPE dependence on laser power, which is measured in milliwatts of average power just after G1. THG emission is the integrated signal curve from 500nm to 545nm in the region of the notch filter. Continuum emission is the integrated signal curve from 400nm to 900nm excluding the signal in the region of the notch filter. The log-log plot indicates a THG line slope of 2.8 with $r^2 = 0.996$. The continuum emission line has a slope of 1.95 with $r^2 = 0.992$, which indicates that this continuum emission is a 2P process. Based on the work of Beversluis et al., whom obtained similar characteristics for the emission spectrum of 100nm GNPs at 780nm fs excitation [19], the observed GNP broad-band emission is likely due to intraband transitions of $d$-band electrons into the conduction band. It is not due to heating of the particles, although detailed GNP heating effects are not studied in this work. The total power of the continuum emission is greater than the THG power for the laser powers that were measured.

Next, 20nm, 40nm and 60nm GNPs are analyzed with respect to relative MPE spectral characteristics with an average laser power of 34.8mW and integration time of 0.5 sec or 1.0 sec. Separate substrates are used for each particle size. Figure 8 shows unnormalized spectral curves for four different 20nm, four different 40nm diameter spherical GNPs and one 60nm diameter spherical GNP. The horizontal range from 500nm to 545nm is expanded to show characteristics of the THG peak. Above 545nm, the vertical scale is expanded by a factor of 10. Individual GNPs are identified with A, B, C or D for each particle size. For example, A-40nm is particle A of the 40nm diameter group. Surprisingly, all particles show basically the same continuum emission characteristics with respect to power and spectral shape. In these data, only one curve, which is the second A-40nm curve identified in the legend, had a 1.0sec integration time. The other curves were obtained with a 0.5sec integration time. In order to compensate for the different powers, the 1.0sec integration curve was scaled down by a factor of two. THG peak powers decrease nearly proportional to the particle size. In two curves, SHG peaks were observed, which may be due to proximity of other particles or some type of contamination. The A-40nm curves, for which 0.5sec and 1.0sec integration times are used, show considerably less power that the other three 40nm particles, which may be due to a misalignment.
4. Conclusions

A hyper-NA (2.8) scanning multi-photon microscope is demonstrated using a compact femtosecond laser source at $\lambda = 1.56 \, \mu m$. SHG, THG and 2PE are observed in samples containing cells and gold nanoparticles (GNPs). The samples are mounted on a silicon substrate, which can provide $\sim 2x$ collection efficiency with high reflectance for two- and three-photon signals. High resolution interaction volume is limited to $\sim 0.025$ femtoliters within 100nm of the surface with a spot full-with-at-half maximum of 400nm for the actinic 1.56$\mu$m fs laser wavelength. A strong THG signal is present from the silicon/water interface. MPE observed from differently sized GNPs is studied in detail. Signals from 20nm, 40nm and 60nm diameter GNPs indicate that the MPE is a two-photon effect and is likely continuum emission. Occasionally, a SHG peak is observed in emission profiles. Interestingly, unnormalized spectral curves of the differently sized GNPs indicate similar spectral shape and emission power.

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