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SHORT COMMUNICATION

Adaptive aberration correction in a two-photon microscope

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Summary

We demonstrate aberration correction in two-photon microscopy. Specimen-induced aberrations were measured with a modal wavefront sensor, implemented using a ferroelectric liquid crystal spatial light modulator (FLCSLM). Wavefront correction was performed using the same FLCSLM. Axial scanned (x-z) images of fluorescently labelled polystyrene beads using an oil immersion lens show restored sectioning ability at a depth of 28 μm in an aqueous specimen.

Introduction

It is well known that the presence of aberrations in a microscope system leads to degraded imaging quality and reduced signal level. The causes and effects of aberrations in confocal microscopy have been investigated experimentally by several authors for both reflection modes (e.g. Wilson & Sheppard, 1984; Wilson, 1990) and single-photon (1-p) fluorescence modes (e.g. Gibson & Lanni, 1991; Hell *et al.*, 1993). There are various reasons why aberrations may be present in a system, for example, misalignment of optics or non-optimal operating conditions. In particular, the use of high numerical aperture objective lenses to image specimens through a refractive index mismatch leads to the introduction of spherical aberration. Such aberrations, for example those introduced by a mismatch of refractive index between the immersion medium, the coverglass and the specimen, lower the intensity of the focal spot and increase its size. When focusing into such a specimen with a confocal microscope, the images obtained are dimmer and suffer from degraded lateral and, more importantly, axial resolution.

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Two-photon (2-p) microscopy is becoming widespread as a complementary method to confocal microscopy in the three-dimensional imaging of biological specimens (Denk *et al.*, 1990; Pawley, 1995). 2-p excitation of a fluorophore occurs as a result of the simultaneous absorption of two long-wavelength photons. A suitably excited fluorophore then emits a photon with a wavelength approximately half that of the excitation light. The intensity of the fluorescence emission is proportional to the square of the illumination intensity. The region of fluorescence emission is therefore confined to the focal spot and imaging performance is comparable to that of a confocal single photon system operating at the longer 2-p excitation wavelength. For this reason, 2-p scanning microscopes are often used without a detector pinhole in order to maximize the signal and hence, for a given fluorophore, the linear dimensions of the 2-p point spread function (PSF) are typically twice those of the confocal 1-p PSF (Gu, 1996). The advantages of using 2-p excitation include the facts that losses due to scattering and lower order phase aberrations are reduced and hence imaging deep into scattering or aberrating media is more efficient using 2-p excitation. However, Jacobsen *et al.* (1994) have shown that, when focusing into a fluorescent sea using a high NA lens, such aberrations substantially reduce the 2-p fluorescence intensity even at a few micrometres focusing depth. The effects have also been investigated by de Grauw *et al.* (1999), who have shown the decrease in intensity and resolution when imaging fluorescent beads and biofilms.

The effects of specimen-induced aberrations can, in principle, be removed by the introduction of the conjugate phase aberration into the optical path. This could take the form of static correction plates, such as the binary phase plates demonstrated in Sieracki *et al.* (1995) or dynamic correction using adaptive optics techniques. Adaptive optics generally involves measurement of the

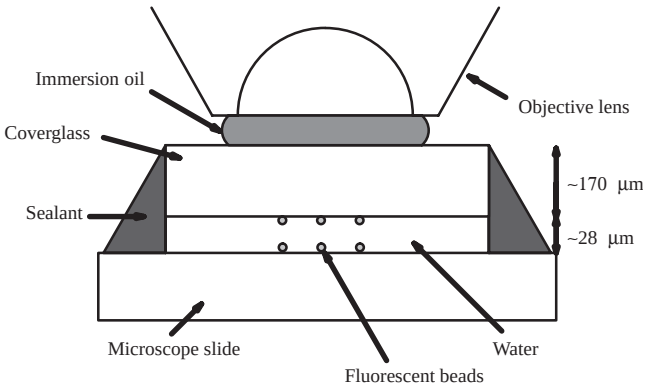

Fig. 1. Schematic diagram of the specimen. The fluorescent beads have diameter of 100 nm and the coverglass has nominal thickness of 170 μm.

induced wavefront aberrations with a wavefront sensor. Compensation is then performed with an adaptive element that pre-shapes the wavefronts of the illuminating beam with an equal but opposite aberration, restoring diffraction limited performance (see, e.g. Tyson, 1991; Love, 2000). In a two-photon microscope, it is not necessary to correct for aberrations induced in the emission path because large area detectors can be employed which act as collectors for the fluorescence light. By continuous measurement of the induced aberrations, the adaptive system is capable of correcting for aberrations as they change, for example, whilst scanning through a specimen. Albert *et al.* (2000) demonstrate a related system using a deformable mirror as a path-length correction element to restore the second-harmonic intensity in a non-linear crystal. In this paper we demonstrate what is, to our knowledge, the first example of adaptive aberration correction in 2-p microscopy by measuring the specimen-induced aberrations and pre-shaping the wavefronts in the input beam.

Method and results

A specimen was prepared by placing small amounts of a suspension of fluorescently labelled polystyrene spheres on both a microscope slide and a coverglass. These were left to dry so that the spheres adhered to the surfaces. The coverglass was then placed on the microscope slide with a thin layer of water separating the two; this is shown schematically in Fig. 1. The thickness of the water layer was measured, taking into account the focal shift, as 28 μm. The fluorescent spheres were 100 nm diameter carboxylate modified microspheres (excitation/emission = 365/515 nm, Molecular Probes, Oregon, U.S.A.).

The optical system is shown in Fig. 2. A mode-locked titanium sapphire laser beam (Coherent Mira) was expanded then coupled into a wavefront generator, which we used as the wavefront correction element. This wavefront generator, based around a Displaytech FLCSLM (Displaytech, Longmont, CO, U.S.A.) is described in previous papers (Neil *et al.*, 1998; Neil *et al.*, 2000a). It can produce arbitrary and accurate phase and amplitude wavefronts at the expense of reduced optical throughput. The output from the wavefront generator was coupled into a specially modified port in a commercial microscope, which allowed full use of the internal filter wheel and photodetector assemblies. The detector pinholes were opened fully in order to maximize the signal. The objective lens was a Plan Neofluar, oil-immersion, 63×, 1.25 NA lens with coverglass correction. Scanned images were taken using the software provided with the microscope.

Any aberration can be considered as a summation of Zernike aberration modes (Born & Wolf, 1975). Typically, only a small number of these modes are required to describe the aberrations present in a microscope system (Booth *et al.*, 1998). We used a method of wavefront sensing which allows sequential measurement and correction of each Zernike mode (Neil *et al.* 2000b, c). In its normal configuration, the FLCSLM aberration generator creates a

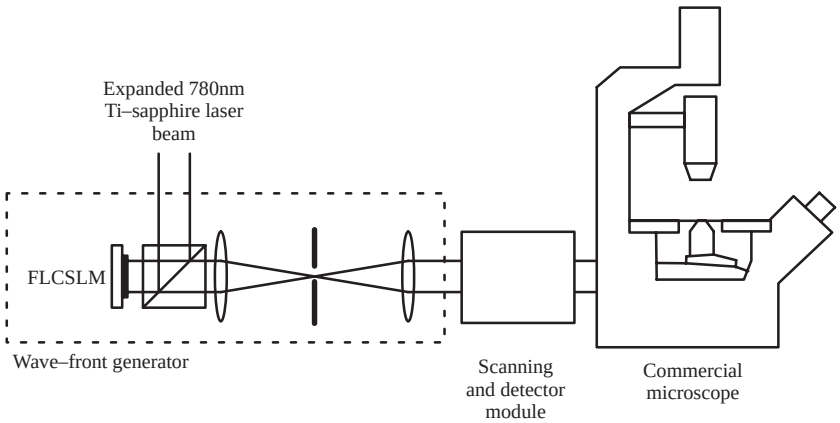

Fig. 2. The experimental set-up.

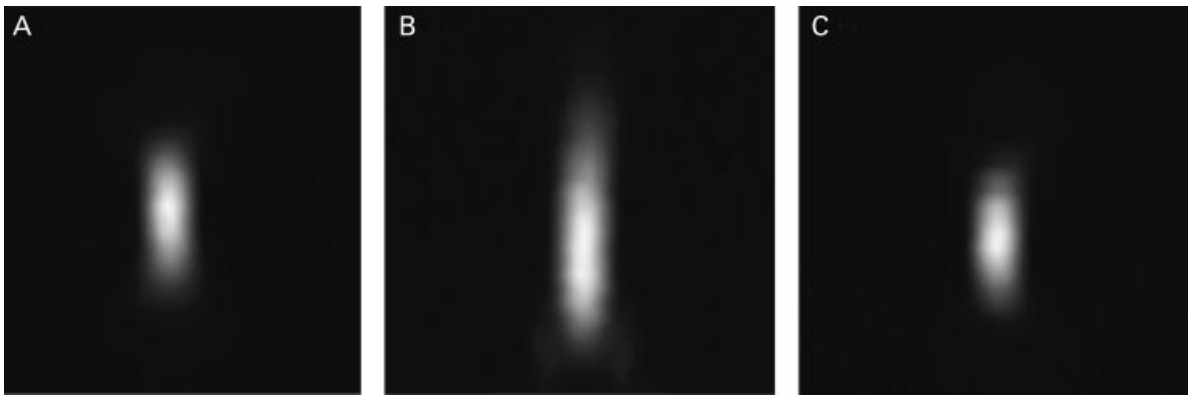

Fig. 3. *x-z* scans of single 100 nm fluorescent beads (a) just below the coverglass, no correction (b) on the surface of the microscope slide beneath 28 μm of water with no correction, (c) on the surface of the microscope slide, corrected. The images are 5 μm × 5 μm in dimension.

single focal spot in the specimen containing a correction aberration. For diffraction limited imaging, this correction should be the conjugate of any induced aberrations. It is also possible to configure the FLCSLM so that a pair of adjacent focal spots is generated, each spot containing an equal but opposite bias aberration in addition to any correction aberration. As this spot pair is scanned across a single fluorescent bead, two bead images are produced. Any difference in brightness between the two bead images indicates the presence of that particular bias mode in the induced aberration. The correction element is then adjusted to remove that particular aberration mode, so that the two bead images are of equal brightness. By sequentially applying different low-order Zernike bias modes and adjusting the corresponding mode in the correction aberration, we arrive at the optimum correction. Reconfiguring the FLCSLM to produce a single spot containing this correction aberration then using the scanning microscope as normal results in a near diffraction limited image.

Results are presented in Fig. 3. Figure 3(a) shows an *x-z* image of a bead situated just below the coverglass with no correction applied. Figure 3(b) shows a bead attached to the microscope slide with no correction applied. It is possible to see the effects of the induced aberrations, including the spherical aberration introduced by the refractive index mismatch between the coverslip and the water layer. The aberrations create an elongated PSF, which causes blurring of the bead image in the axial direction. Figure 3(c) shows the same bead as Fig. 3(b) but with the correction applied so that diffraction limited focusing is restored. In each case, the illumination power available at the objective was approximately 2 mW. The relative brightness of each image has been adjusted to show detail, although it should be noted that Fig. 3(b) was dimmer than Figs 3(a) and (c). The correction aberration applied when taking Fig. 3(c) consisted of seven low-order Zernike modes. Table 1 shows the amplitudes of these modes, along with the names of the aberrations they represent. In Table 1, we use the notation and definition of the Zernike polynomials, as detailed in Neil *et al.* (2000b).

Table 1. The Zernike modes and the amplitudes used in the correction for Fig. 3(c).

Zernike mode	Amplitude	Description
$Z_{2,2}$	− 0.20	Astigmatism (1st order)
$Z_{3,1}$	− 0.20	Coma (1st order)
$Z_{3,-1}$	0.40	Coma (1st order)
$Z_{4,0}$	− 1.30	Spherical (1st order)
$Z_{5,-1}$	0.12	Coma (2nd order)
$Z_{6,0}$	− 0.34	Spherical (2nd order)
$Z_{8,0}$	− 0.08	Spherical (3rd order)

Conclusion

We have demonstrated how adaptive optics can be used to compensate for specimen-induced aberrations in two-photon microscopy. Using a new method of wavefront sensing, implemented with a FLCSLM, we measured and compensated for aberrations induced by a mismatch in refractive index. This is the first time that this form of wavefront sensing has been used in microscopy. A drawback of this particular implementation is the inefficient use of laser light. The wavefront generator described in this paper had an optical throughput of around 1–2% at a wavelength of 780 nm. This was due to a combination of fundamental and device-specific factors. We believe that by careful optimization of the device parameters optical throughputs of up to 10% are practically achievable. However, this level of efficiency is likely to be adequate for single point scanning

2-p microscopy where there is often a surplus of laser power available. A more efficient implementation of the aberration correction system might use components such as deformable mirrors or nematic liquid crystals.

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