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Adaptive optics in microscopy

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The imaging properties of optical microscopes are often compromised by aberrations that reduce image resolution and contrast. Adaptive optics technology has been employed in various systems to correct these aberrations and restore performance. This has required various departures from the traditional adaptive optics schemes that are used in astronomy. This review discusses the sources of aberrations, their effects and their correction with adaptive optics, particularly in confocal and two-photon microscopes. Different methods of wavefront sensing, indirect aberration measurement and aberration correction devices are discussed. Applications of adaptive optics in the related areas of optical data storage, optical tweezers and micro/nanofabrication are also reviewed.

Keywords: adaptive optics; aberrations; confocal microscopy; multiphoton microscopy; optical data storage; optical tweezers

1. Introduction

Optical microscopes are essential tools in many scientific fields. In the life sciences, they are widely used for the visualization of cellular structures and subcellular processes. Confocal and multiphoton microscopes are particularly important in this respect as they produce three-dimensional images of volumetric objects. However, the resolution of these microscopes is often adversely affected by the optical properties of the specimen itself. Spatial variations in the refractive index of the specimen introduce optical aberrations that compromise image quality. This is a particular problem when imaging deep into thick biological specimens. Ultimately, the aberrations restrict the amount of specimen that can be observed by the microscope, the depth being limited to a few cellular layers near the surface. This is a serious limitation if one wants to observe cells and their processes in their usual environment, rather than in the unnatural surroundings of a microscope slide.

Until a few years ago, this was a problem that many people knew about, but nobody could fix. Recently, researchers have started to use adaptive optics, a technique borrowed from astronomy, to measure and correct the aberrations and restore the optimum resolution of these systems. The techniques were originally developed for astronomical and military purposes, for stabilizing and de-blurring telescope images of stars and satellites affected by the aberrations caused by atmospheric turbulence (Tyson 1991; Hardy 1998). Adaptive optics technology is

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now being developed for more down-to-earth reasons, in smaller scale applications such as ophthalmic imaging and communications. Naturally, the same principles can be applied to microscopy.

The most widely used optical microscopes for high-resolution biomedical imaging are the scanning confocal and two-photon fluorescence microscopes (Wilson 1990; Gu 1996; Pawley 2006). One of the major strengths of these methods is their ability to provide three-dimensional images of volumetric specimens. In the confocal microscope, the three-dimensional resolution arises through the use of a pinhole situated before the photodetector. This pinhole obscures out-of-focus light and allows only light from the focus to be efficiently detected. The two-photon microscope relies upon the process of two-photon excitation fluorescence, in which a fluorophore is excited by the simultaneous absorption of two photons. As the efficiency of two-photon excitation process is proportional to the square of the illumination intensity, fluorescence is only efficiently generated in the focal spot, where the intensity is highest. The two-photon microscopy therefore exhibits intrinsic three-dimensional resolution without the need for a detector pinhole (Sheppard & Gu 1990).

Aberrations affect all types of microscopy, but the effects are particularly significant for both confocal and two-photon microscopy, causing a reduction in signal level, contrast and resolution. By correcting the aberrations, adaptive optics can improve the image quality or, in the best case, restore the optimum performance of the microscope. Several such adaptive optics systems have been implemented in microscopes and other closely related optical engineering systems. The requirements of these systems have led to the development of several new techniques. In this review, I present an overview of aberrations in microscopy and describe recent developments in microscope adaptive optics.

2. Aberrations in microscopy

(*a*) *Description of wavefront aberrations*

A high numerical aperture (NA) objective lens transforms planar wavefronts that pass through the lens pupil into spherical wavefronts in the focal region (figure 1). As the light passes through the optical system and the specimen, aberrations can be introduced that distort the wavefronts from this ideal planar or spherical form. These aberrations can be modelled as phase variations in the pupil of the objective lens. In principle, the aberrations are compensated by introducing an equal but opposite phase aberration into this pupil (or its optical conjugate) using an appropriate correction element. In order to reduce the amount of information required to specify aberrations, it is useful to represent them as an expansion in orthogonal functions. In this way, the aberration can often be accurately represented by a short sequence of function coefficients. In systems with circular pupils, the Zernike circle functions are regularly used as, in addition to their convenient mathematical properties, the low-order Zernike modes correspond closely to traditional aberration terms, such as astigmatism, coma or spherical aberration (Zernike 1934; Born & Wolf 1983; Mahajan 2001). Other sets of modes may also be appropriate, such as the eigenmodes of the correction element (Paterson *et al.* 2000).

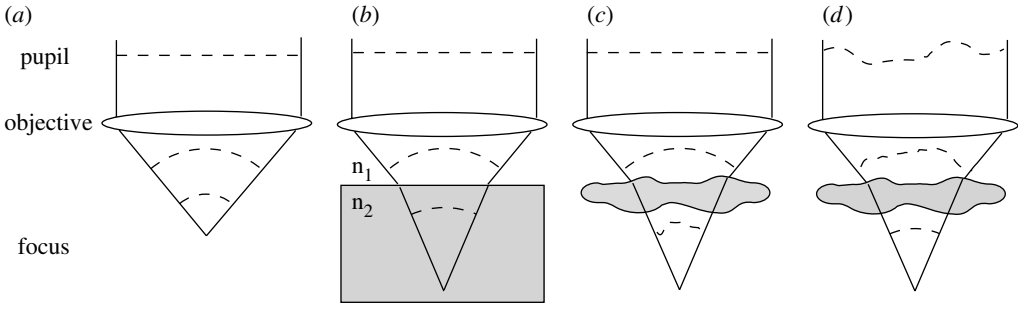

Figure 1. (*a*) Schematic of focusing by a high-NA objective lens. Planar wavefronts in the pupil are converted into convergent spherical wavefronts in the focus. (*b*) The effects of focusing through a refractive index mismatch, where refraction at the interface distorts the wavefronts. (*c*) Focusing through a complex specimen, where refractive index variations introduce aberrations. (*d*) The principle of aberration correction—a conjugate phase introduced in the pupil is cancelled out by the specimen-induced aberrations.

(*b*) *Sources of aberrations*

One of the most studied specimen geometries is that of the planar refractive index mismatch, where the light is focused through a planar interface between two media with different refractive indices (figure 1*b*). This structure is common to many applications: it occurs in microscopy when focusing using an immersion objective, through a cover glass, or into a specimen-mounting medium. The effects of such refractive index mismatch on focusing were first investigated by Sheppard and colleagues (Sheppard & Cogswell 1991; Sheppard & Gu 1991). A comprehensive review of further studies pre-1998 is provided by Egner & Hell (1998). The primary effects of the refractive index mismatch are focal shift and spherical aberration that are proportional to the focusing depth; these can jointly be represented by a series of rotationally symmetric Zernike modes (Török *et al.* 1995; Booth *et al.* 1998; Booth & Wilson 2000). In microscopy, the spherical aberration induced by the refractive index mismatch of the cover glass is often removed by incorporating a correction aberration into the objective lens design. Further benefit is obtained through the use of water immersion objective for imaging aqueous specimens. Some of these objective lenses are equipped with adjustable cover glass correction, to enable the use of different cover glass thicknesses, although careful use is required to ensure correct operation (Schwertner *et al.* 2005).

A more significant problem is presented by the complex aberrations introduced by the optically inhomogeneous structure of biological specimens (figure 1*c*). Small but measurable variations have been found in direct measurements of the refractive indices of biological materials and organelles (Bolin *et al.* 1989; Tearney *et al.* 1995). These variations can lead to significant aberrations when focusing through thick specimens. Characterization of these aberrations is essential for the understanding of the requirements for a microscope adaptive optics system. Schwertner *et al.* (2004*a*,*b*) used an interferometer to directly measure specimen-induced aberrations and quantified the results in terms of Zernike function coefficients (figure 2). The aberrations can also be inferred if one knows the refractive index profile of the specimen.

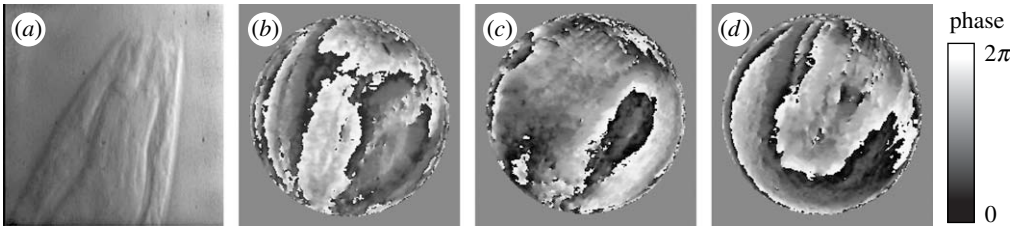

Figure 2. (*a*) Specimen-induced aberrations from a *Caenorhabditis elegans* specimen (transmitted light, dimensions 60×60 μm). (*b–d*) The three example images show the phase in the objective lens pupil. These phase data are reconstructed from the direct interferometric measurements of 633 nm wavelength light focused through the specimen using a 1.2 NA water immersion objective (Schwertner *et al.* 2004*b*).

Kam *et al.* (2001) used Normarski differential interference microscopy to reconstruct this profile. Refractive index profiles have also been reconstructed using tomographic holographic microscopy (Charrière *et al.* 2006).

It should be noted that not all aberrations are caused by the specimen. Although great effort goes into the design of high-resolution microscopes to ensure that they operate at the diffraction limit, aberrations caused by imperfections in the optical system can still be present. For example, if a lens is used off-axis or with the wrong image conjugate, aberrations will be present to some degree (Born & Wolf 1983; Sheppard & Gu 1991). They may also arise when the system is not used under design conditions, for example with wrong wavelength or incorrect temperature (Juškaitis 2006).

(*c*) *Effects of aberrations*

There is a complex relationship between the phase aberrations in the pupil and the form of the focal spot. However, broadly speaking, aberrations lead to spreading of the focus both in the lateral plane and, more importantly, an elongation along the optic axis. This is accompanied by a reduction in the focal intensity. The distortion of the focal spot reduces the resolution of the system and causes a blurring between adjacent planes along the optic axis. The drop in intensity leads to a loss of optical efficiency and image contrast. Multiphoton processes are strongly affected by aberrations as the absorption probability is nonlinearly dependent on the focal intensity. In the confocal microscope, the fluorescence generation decreases in proportion to the focal intensity. However, the imaging of this fluorescence onto the detector pinhole is also affected, so the detected signal falls more rapidly due to the compound effect of aberrations in both the illumination and detection paths. Therefore, signal levels in confocal and two-photon microscopes are affected to a similar degree by aberrations (Gu & Sheppard 1995; Ganic *et al.* 2000; Booth & Wilson 2001). Signal levels can be restored by increasing the illumination laser power to compensate for the lower focal intensity, although this does nothing to restore the resolution. It also has the disadvantages of increasing photobleaching and photo-toxic effects—this is of particular importance in live cell imaging, where the lowest exposures are desirable (Hoebe *et al.* 2007).

(*d*) *Constructive use of aberrations*

It would be wrong on the part of the reader to think that aberrations are unconditionally detrimental to microscopy. Indeed, there are examples where aberrations have been put to good use in enhancing microscope images. It is possible to adjust imaging properties by introducing a phase pattern into the lens pupil (Neil *et al.* 2000*c*). This effect is most beneficial when combined with nonlinear methods, such as stimulated emission depletion (STED) microscopy (Hell 2007). In another method known as wavefront coding, aberrations are intentionally introduced in order to extend the depth of the focus of microscopes (Tucker *et al.* 1999). When imaging thick specimens, it is possible that light from out-of-focus planes contributes a significant background signal. While aberrations strongly affect in-focus image quality, they have little effect on out-of-focus background. This observation has allowed Leray & Mertz (2006) to remove the background fluorescence in multiphoton microscopy by subtracting a heavily aberrated image from an aberration-free image.

3. Adaptive optics for microscopy

(*a*) *Implementations of adaptive optics in microscopy*

One of the earliest implementations of adaptive optics in microscopy was the use of tip/tilt correction in a transmission confocal microscope (O'Byrne *et al.* 1999). The major problem encountered here is that the position of the spot at the detector is shifted laterally by structures in the specimen. The authors proposed using a tilting mirror in the transmission path to maintain the position of the focal spot on the detector. Unlike this system, most microscopes are used with *epi* illumination, with illumination and detection light passing through the same objective lens. In this epi configuration, tip/tilt and defocus aberrations are self-correcting, hence the focus is always conjugate to the detector. It should be noted, however, that this phenomenon does lead to image distortion (Schwertner *et al.* in press).

The confocal microscope provides maximum resolution and signal level when both illumination and emission paths are diffraction limited. As the illumination and fluorescence light both pass through the specimen, aberrations will be introduced into both the paths. It is clear that to restore diffraction-limited operation, aberration correction must be performed in both the paths. This dual path correction is readily implemented using a single deformable mirror (DM), a configuration that has been employed successfully in confocal fluorescence microscopes (Booth *et al.* 2002*b*; Wright *et al.* 2005; figure 3*a*). As two-photon microscopes are normally used without a detector pinhole, aberrations in the detection path are unimportant as they have no effect on the resolution or signal level. Therefore, an adaptive two-photon microscope needs only incorporate aberration correction in the illumination path. Such systems have been implemented using DMs (Sherman *et al.* 2002; Marsh *et al.* 2003; Rueckel *et al.* 2006) and liquid crystal spatial light modulators (Neil *et al.* 2000*b*).

In addition to scanning microscopes, adaptive optics has also been implemented in imaging microscopes. Adaptive correction for the imaging of two-dimensional objects has been demonstrated in a transmission microscope

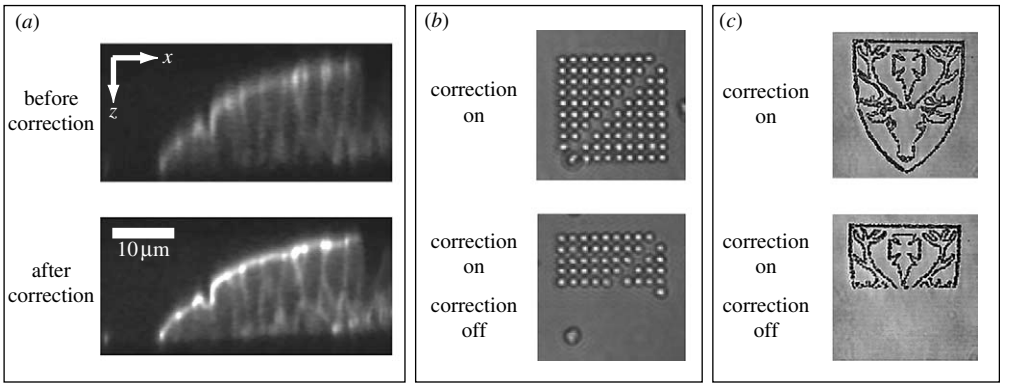

Figure 3. (*a*) Adaptive confocal fluorescence microscope images of labelled mouse intestine. Aberrations were determined using sequential modal wavefront sensing (Booth *et al.* 2002*b*). (*b*) Transmission microscope images of simulated bit data recorded as voids at a depth of 100 μm in a multilayer optical data storage device (Booth *et al.* 2006*b*). The exposure for the upper and lower images was identical, except that the aberration correction was switched off for part of the lower pattern. Image size 20 μm. (*c*) Aberration correction for optical micro-machining of channels in a polymer substrate (Schwertner *et al.* 2006). The lower image shows that the machining process is only effective with aberration correction. Image size 80 μm.

(Débarre *et al.* 2007). In a different application, predictive aberration correction has been used to extend the field of view of a microscope. Potsaid *et al.* (2005) combined an objective lens designed to have residual aberrations with a DM that compensated aberrations over a sub-region of the field of view. In this way, they were able to maintain diffraction-limited performance for a sequence of sub-images over a much extended field.

(*b*) *Wavefront sensing and aberration measurement*

Several types of wavefront sensing have been developed for adaptive optics, the most prominent methods being the Shack–Hartman wavefront sensor and interferometric sensors (Tyson 1991; Hardy 1998). These methods require a point-like reference source, such as the distant guide star used in astronomical systems, to produce a well-defined wavefront. In adaptive microscopy, the situation is more complicated as the three-dimensional structure of the specimen means that the reference source is generally far from point-like. In effect, the wavefront sensor would receive a multitude of wavefronts, each emanating from different parts of the specimen. Therefore, direct wavefront sensing is not straightforward in microscopy.

Further problems arise if reflected or scattered light is used as the reference source, as the coherent interaction of the light and the specimen structure can cause ambiguity in the wavefront measurement. This effect was discussed by Artal *et al.* (1995) in the context of ocular imaging. For specular reflection from a planar object, odd (asymmetric) aberrations in the illumination and reflection paths are cancelled, whereas the even (symmetric) components add up. For diffuse scattering, both odd and even aberrations can be measured. This problem does not occur for fluorescence emission, as the phase information from the illumination wavefront is always lost in the incoherent fluorescence process.

Most of the adaptive microscope systems so far implemented have not employed a wavefront sensor but have used indirect methods of aberration measurement. This approach requires relatively minor modification of the microscope, i.e. the inclusion of an adaptive optical element, and avoids the wavefront sensor pitfalls described previously. In general, this indirect approach involves the maximization of the photodetector signal using an appropriate optimization scheme. Several of these implementations have used stochastic methods based upon genetic algorithms or hill-climbing algorithms (Albert *et al.* 2000; Sherman *et al.* 2002; Marsh *et al.* 2003; Wright *et al.* 2005). These methods tend to require a large number of iterations that might not be practical for some applications where high speed or low exposure is required. Model-based approaches based upon modal wavefront sensing have shown better performance, requiring fewer measurements than model-free methods (Booth *et al.* 2002*b*; Booth 2006, 2007). Stochastic gradient descent algorithms (Vorontsov 2002) have been used in other areas of adaptive optics and might also have useful application in microscopy. Indirect aberration measurement through phase retrieval from images has also been suggested (Hanser *et al.* 2004).

Direct wavefront sensing can be applicable to microscopy if combined with a method for excluding out-of-focus light. For example, the modal wavefront sensor introduced by Neil *et al.* employs pinholes that behave much like the detector in a confocal microscope (Neil *et al.* 2000*a*; Booth *et al.* 2002*a*). Feierabend *et al.* (2004) have implemented a wavefront sensor that uses coherence gating to exclude the effects of out-of-focus light. This is a promising method for wavefront sensing in thick scattering specimens, such as brain tissue (Rueckel *et al.* 2006). However, in both cases, measurements are still to a degree dependent upon the specimen structure.

(*c*) *Aberration correction devices*

The commercial availability of practical, affordable correction devices has probably been the main driving factor in adaptive optics for microscopy. The development of liquid crystal (LC) panels for displays has led to spatial light modulators for research applications. These have been demonstrated in adaptive microscopy (Neil *et al.* 2000*b*), but have not been widely applied. The main drawbacks of LC devices are their polarization and wavelength dependence, which are not compatible with fluorescence microscopy. LC devices have, however, found wider application in optical data storage and optical trapping (see §4).

Microelectrical mechanical systems (MEMS) technology has enabled the production of a number of DM systems. These are particularly useful for fluorescence imaging, where the high optical efficiency of the mirror ensures that optical losses are low. The most widely used DMs have continuous reflective surfaces, although segmented MEMS mirrors are also becoming available (Dagel *et al.* 2006). Electrostatically actuated membrane DMs consist of a thin membrane with a reflective coating mounted above an electrode array (Vdovin *et al.* 1999). By applying a potential between the membrane and the electrode, the DM surface shape can be pulled towards the electrodes. A transparent electrode can also be included above the membrane to permit push–pull deformations (Bonora & Poletto 2006). Other electrostatically actuated MEMS DMs have been based upon microstructured silicon (Bifano *et al.* 1999). Here a supporting structure below

the reflective membrane ensures that the influence of each actuator on the DM shape is spatially more confined than for a membrane DM. Piezo-electric actuators have been employed in DMs both using direct actuation of the mirror surface (Simonov *et al.* 2006) and through curvature control using bimorph actuators (Dainty *et al.* 1998). Magnetically actuated DMs have also been demonstrated (Fernandez *et al.* 2006). These DMs have been complemented by more exotic technologies such as thermally actuated (Vdovin & Loktev 2002), fluidic (Vuelban *et al.* 2006) and ferromagnetic devices (Laird *et al.* 2006).

Given the wide range of DMs available, the selection of an appropriate device for a particular application is a non-trivial task. While properties such as cost, size, speed and robustness are relatively self-explanatory, the aberration correction ability of a device is harder to evaluate. The standard quantities specified by manufacturers, such as maximum stroke or number of actuators, are not themselves useful guides to the compatibility of a device with an aberration correction task—more is not necessarily better. Several methods have been developed to characterize DMs and determine the range of correctable aberration modes (Zhu *et al.* 1999; Fernandez & Artal 2003; Booth *et al.* 2005) and the performance for particular correction tasks (Paterson *et al.* 2000; Dalimier & Dainty 2005).

4. Other applications of adaptive optics

The optical systems used in high-resolution microscopy are also central to other applications in optical and photonic engineering. These include data storage, optical trapping and micro/nanofabrication. All of these applications require the resolution provided by the tight focusing of light using high-NA objective lenses. As in microscopy, these methods suffer from aberrations introduced as the focused light passes through different parts of the specimen or substrate. The efficiency and accuracy of all of these processes are detrimentally affected by aberrations and the use of adaptive optics has great potential.

Much of the recent research into three-dimensional optical data storage has used multiphoton processes to record data and confocal microscopes for read-out. As these systems invariably involve focusing through a refractive index mismatch, aberration control is an important consideration in system design and the use of adaptive aberration correction has been explored. In these systems, the recording substrate is predominantly isotropic, so aberrations can be predicted from the focusing depth, obviating the need for direct aberration measurement. LC devices have been used in single or dual recording layer systems to correct spherical aberration due to the disc thickness and coma caused by disc tilt (Ohtaki *et al.* 1999; Somalingam *et al.* 2004; Knittel *et al.* 2005). The aberrations become more significant in three-dimensional storage where focusing depths are much larger. Early work in three-dimensional memory involved the compensation of spherical aberration by adjusting the tube length of the objective lens (Day & Gu 1998). Neil *et al.* (2002) employed an LC device to record data at a depth of 1 mm in a high refractive index medium. Booth *et al.* (2006) have demonstrated a DM-based system for aberration correction in the recording and read-out of multilayer polymer media (figure 3*b*).

Optical tweezers have been widely applied for the manipulation of small particles on the micrometre scale (Grier 2003; McGloin 2006). The effectiveness of optical tweezers is compromised by aberrations (Rohrbach & Stelzer 2002; Ganic *et al.* 2004; Roichman *et al.* 2006; Vermeulen *et al.* 2006) and adaptive optics have been applied by various groups to improve performance. Tube length correction was employed by Ke & Gu (1998) to compensate for refractive index mismatch-induced aberrations. Ota *et al.* (2003) used a DM to manually adjust spherical aberration introduced when trapping in an aqueous medium using an oil immersion objective. Theofanidou *et al.* (2004) optimized the two-photon fluorescence signal from a trapped fluorescent bead to increase trap strength. Aberrations have also been compensated in a system employing a LC spatial light modulator as a holographic element (Wulff *et al.* 2006).

Optical methods of nanofabrication have been used to manufacture polymer, ceramic and metallic structures with feature sizes as small as 100 nm (Serbin *et al.* 2003; Sun & Kawata 2004; Ishikawa *et al.* 2006). This sub-wavelength resolution is achieved through the use of nonlinear, thresholded optical processes. Thresholded multiphoton processes have also been employed to machine structures in high refractive index media for the manufacture of photonic crystals (Wong *et al.* 2006; Zhou & Gu 2006). As these methods require focusing between materials of different refractive indices, they suffer from aberrations that can reduce the focal intensity. If this drops below the threshold level, the fabrication effect can be lost entirely. Adaptive optics can be used to maintain focal spot quality even when focusing at depth in the fabrication substrate (Schwertner *et al.* 2006; figure 3*c*).

5. Future outlook

The principles of adaptive optics for microscopy and photonic engineering have been demonstrated in a number of implementations and applications. However, there are still many technical challenges to be met before adaptive optics becomes a standard feature. Studies have shown that specimen-induced aberrations can change significantly over a microscope's field of view (Schwertner *et al.* 2004*a*,*b*), so real-time adaptive optics for confocal and two-photon microscopes would be desirable. This may only be possible with improved aberration measurement schemes and faster correction devices—current DMs are limited in speed to a few kilohertz, whereas the fastest pixel rates in commercial microscopes can be in excess of 100 kHz. Advances could be made in correcting field-dependent aberrations through the use of multi-conjugate adaptive optics, where multiple adaptive elements are employed (Kam *et al.* 2007). This would also enable the use of adaptive optics in widefield microscopes, including the widefield sectioning microscopes that produce confocal-like images (Conchello & Lichtman 2005). There is also much scope for the use of adaptive optics in emerging microscopy methods. This is especially the case in microscopies that rely upon nonlinear effects, such as third harmonic generation (Débarre *et al.* 2006), coherent anti-Stokes Raman microscopy (Zumbusch *et al.* 1999) or STED microscopy (Hell 2007). Perhaps one of the major benefits of microscope adaptive optics will be for *in vivo* tissue

imaging, as aberration correction provides better image contrast with the use of lower illumination intensities, correspondingly lower phototoxicity and improved specimen viability.

In three-dimensional optical data storage, the predominant challenge is the production of compact, low-cost and robust adaptive devices that can be incorporated into an optical drive. The most promising candidates seem to be LC devices that are currently being used in optical disc systems (Knittel *et al.* 2005). Most of the current work in aberration correction for optical tweezers has concerned the compensation of low-order spherical aberration. For trapping within complex biological specimens, it will be necessary to develop more advanced adaptive schemes. Optical fabrication methods will be more affected by aberrations as the size and complexity of the fabricated structures are increased. Again advances in microscope adaptive optics will help maintain the precision of the fabrication systems.

Images in figures 2 and 3 are reproduced with the kind permission of M. Schwertner and T. Wilson. The author is grateful to E. Botcherby, D. Débarre, R. Juškaitis, M. Schwertner and T. Wilson for numerous useful discussions concerning this material.

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