Modelling the application of adaptive optics to wide-field microscope live imaging

ZVI KAM*, PETER KNER†, DAVID AGARD†,‡ AND JOHN W. SEDAT†
*Weizmann Institute of Science, Rehovot 76100, Israel
†Department of Biochemistry and Biophysics, University of California at San Francisco, 600 16 Street, San Francisco, CA 94158, W.M. Keck Advanced Microscopy Laboratory, U.S.A.
‡Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA 94143, U.S.A.

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Summary

Wide-field fluorescence microscopy is an essential tool in modern cell biology. Unfortunately the image quality of fluorescence microscopes is often significantly degraded due to aberrations that occur under normal imaging conditions. In this article, we examine the use of adaptive optics technology to dynamically correct these problems to achieve close to ideal diffraction limited performance. Simultaneously, this technology also allows ultra-rapid focusing without having to move either the stage or the objective lens. We perform optical simulations to demonstrate the degree of correction that can be achieved.

Introduction

Fluorescent light microscopy of live cells offers cell biologists the opportunity to study molecular and cellular mechanisms in action. Genetic tagging of proteins, especially rare ones, requires imaging with the best three-dimensional resolution using the highest possible numerical aperture objectives, efficient photon collection, minimal photo-damage, sufficient working distances for thick samples, and minimal sample perturbation during fast live data collection. In practice, all of these requirements cannot be met, and cell biologists must compromise on these aspects of fluorescence imaging. We test the incorporation of adaptive optics to come much closer to achieving all of these objectives.

Fluorescence wide-field microscopy provides the optimal methodology for harvesting the most photons in a given optical configuration. Photon efficiency is of critical importance for in vivo imaging and requires high numerical aperture objectives, which use an immersion medium, such as oil, glycerol or water. However, the index of refraction of living tissue is different than any of these immersion media. Therefore, imaging into live samples degrades the resolution, contrast and peak intensity of the image very rapidly with depth (Hiraoka et al., 1990; Gibson & Lanni, 1991; Kam et al., 1997; Kam et al., 2001; Hanser et al., 2002). This degradation is caused by depth-dependent aberrations (most significantly, spherical aberrations). Aberrations significantly degrade the performance of deconvolution algorithms routinely applied for three-dimensional wide-field image reconstruction with high NA objectives (Swedlow et al., 2002). Using deconvolution algorithms the signal-to-noise ratio and resolution, particularly in the axial direction, are enhanced by pushing the out-of-focus light back to its true three-dimensional source (Swedlow et al., 1997; Swedlow & Platani, 2002; Swedlow, 2003). Aberrations make it impossible to faithfully reconstruct the source of the out-of-focus light. The depth aberration problem is fundamental for laser scanning microscopes as well, particularly two-photon microscopy, causing loss of signal intensity (Marsh et al., 2003; Tsai et al., 2005) and for laser tweezers applications, causing loss of beam holding forces (Theofanidou et al., 2004). Finally, the presence of spherical aberrations inhibits the application of structured illumination techniques which otherwise have the ability to double the three-dimensional optical resolution in light microscopy (Gustafsson, 2000; Gustafsson et al., 2005).

The image of a point source, known as the point spread function (PSF), provides an effective means to characterize aberration-induced image distortions. Depth-dependent aberrations scale roughly as the difference in the refractive indices of the immersion medium (n) and the sample (n′) multiplied by the depth within the sample (see Eq. 2 and its Taylor expansion for small (n′−n) below). Thus
with an immersion oil of refractive index, \( n = 1.518 \), a point image 50 \( \mu \text{m} \) below the coverslide in glycerol (\( n = 1.4746 \), refractive index difference, \( \Delta n = 0.0434 \)) has similar distortions as a point image 12 \( \mu \text{m} \) inside a volume of a typical buffer (\( n = 1.341, \Delta n = 0.177 \)). The corresponding peak intensity is about four times lower than would be detected for an identical point source right under the coverslide. For a water immersion objective imaging into tissue (\( n = 1.41, \Delta n = 0.077 \)), comparable distortions will appear at 28 \( \mu \text{m} \) depth. Aberrations can be corrected by adjusting the refractive index of the immersion medium (Hiraoka et al., 1990) by adjusting special collars on objectives, or by inserting additional adjustable optics into the microscope imaging tube (Kam et al., 1997).

More generally, spherical and other aberrations can be understood to arise from differences in optical path at different locations in the optical wavefront (Ross, 1954). Given a point source imaged at different focal planes, it is possible to reconstruct wavefront amplitude and phase variations in the back aperture of the objective lens. This can then be used in a mixed Fourier/real-space deconvolution algorithm to computationally correct for depth-dependent changes in the PSF (Hanser, 2003). The more general case of sample-induced aberrations was addressed in another paper (Kam et al., 2001) using space-variant deconvolution computed from ray-traced PSFs. Aberrations can thus be corrected by modifying optics (Hiraoka et al., 1990; Kam et al., 1997) or by computational approaches (Kam et al., 2001; Hanser et al., 2002). Inevitably, these approaches slow down the image acquisition process and pose a serious computational burden for the reconstruction. However, an alternative approach would be to directly correct image distortions by introducing compensating optical path differences into the optical wavefront. The development of fast adaptive optics (AO) elements which can adjust the optical path length over the aperture opens new possibilities for real-time correction of both depth-dependent and sample-induced aberrations in live sample microscopy. Moreover, AO elements can alter the optical wavefront so that the focal plane is swept throughout the sample without having to move either the objective lens or the stage.

AO has had an extraordinary impact in astronomy (Tyson, 1991; Wizinowich & Bonaccini, 2002) and has recently been applied to medical imaging and confocal microscopy (Albert et al., 2000; Bartsch et al., 2002; Booth et al., 2002; Shirai, 2002; Schwertner et al., 2004). The correction of aberrations in astronomy (and a recent implementation in scanning confocal microscopy) relies on evaluation of the distortion of point sources (bright stars or the confocal exciting beam) and subsequent modification of the AO element to correct for these distortions. The AO approach described here, in contrast to the astronomy paradigm, deals with the case when we can define and characterize the aberrations beforehand. This approach allows for faster correction and minimizes bleaching because extra images do not need to be acquired. Here we consider two different modes of applying AO to light microscopy of live samples. In the first mode we insert the AO element in a pupil conjugate plane, allowing dynamic focusing without sample perturbation while correcting spherical aberration on the fly for each depth. In the following simulations, we consider imaging into a water-based sample with an oil-immersion lens to demonstrate the correction possible in the extreme case. Through the use of multiple AO elements, the second mode additionally makes possible correction of local refractive index variations within the sample. In this case, we would utilize Differential Interference Contrast (DIC) imaging to empirically provide the required three-dimensional map of index of refraction variations within the sample (Kam 1998; Kam et al., 2001).

**Methods**

The ray tracing program, described in detail before (Kam et al., 2001), is based on the analytical solution of ray paths and phases in a gradient refractive index medium. With this algorithm a larger integration step can be used for a given accuracy than in the common method of Sharma et al. (1982), therefore cutting the processing time. A fan of homogeneously spread rays within a given aperture cone is generated, traced through a refractive index medium, mapped onto a three-dimensional grid, and the emerging wavefront phases at infinity are used to evaluate the Strehl ratio as a complex amplitude integral over the wavefront (Hardy, 1998). In this work a fluctuating refractive index medium was simulated by adding three-dimensional Gaussians at random displacements from a three-dimensional grid according to:

\[
n(r) = n_0 + \Delta n \sum G(r - r_\alpha),
\]

where \( G(r) = \exp(-|r/w|^2) \), \( r = (x, y, z) \), \( r_\alpha = (i + p_i)1_x + (j + p_j)1_y + (k + p_k)1_z \), \( i, j, k \) are integers, \( p_i, p_j, p_k \) are uniform random variables between 0 and 1 and \( 1_x, 1_y, 1_z \) are vectors along \( xyz \) spanning a three-dimensional grid with spacing proportional to \( w \), the Gaussian width. Various dimensions and refractive index contrasts were generated and ray traced. The reported results here correspond to about 500 Gaussians at average spacing of 3\( w \). To calculate the effects of adaptive optical elements, the rays emerging from the medium are ‘relayed’ by ideal lenses as described in Figures 2 and 4, and treated by one or several adaptive optical devices by tracing to their planes and shifting the phases according to the position they hit.

The presentation of a finite slice of the medium by a single conjugated adaptive optical plane followed one of several options: Parallel-sum means integrating the optical path of the slice along the optical axis \( z \) (and perpendicular to the plane) as a function of position \( XY \). Fan-sum performs the optical path integration along rays emerging from the origin. For numerical apertures greater than 1.2, light rays inside the sample travel at angles greater than 45° with the optical axis. Thus there is a significant difference between the parallel-sum and the
Fan-sum methods when using a small number of conjugates. The Tokovinin option (see Tokovinin et al., 2000) ‘blurs’ each slice by an amount increasing with distance from the conjugate plane, and in proportionality to the field of view.

The Zemax ray tracing program (Bellevue, WA 98004-8017) was applied to the Nikon objective described in Yamaguchi (2003). The adaptive element was approximated by expanding Eq. (3) in terms of the first five radial Zernike coefficients.

Results

Correction of depth aberration for water-immersed samples imaged with oil-immersion objectives

The three-dimensional image of a point source, the PSF, can be derived theoretically as a function of depth. The shift in optical path, \( \Delta OP \), from a source in a depth \( D \) of sample medium with refractive index \( n' \) can be analytically expressed (Booth et al., 1998) as:

\[
\Delta OP = D(n' \cos \theta' - n \cos \theta) \sim D(n' - n)/ \cos \theta, \quad (2)
\]

where \( \theta' \) is the angle in the sample medium, and \( \theta \) is the angle in the immersion liquid with refractive index \( n \), (see Fig. 1). From the relation between ray angle and position in the back pupil plane, the PSF can be calculated as the Fourier transform of the back pupil plane wavefront. Theoretical calculations agree with experimental measurements of the PSF shape (Gibson & Lanni, 1991; Booth et al., 1998). Since the phase shift depends on depth and ray angle alone, it can be corrected at each focal plane (depth) for the entire imaged field by introducing a phase correction for each ray at the back pupil plane, where its position corresponds to an angle of emergence from the sample.

In Figure 2a we show a schematic microscope consisting of only the significant optical elements (an infinity corrected objective and tube lens) with an AO element inserted in a plane conjugate to the back pupil plane using appropriate relay optics. In all likelihood the implementation of the AO would be accomplished by a deformable mirror, whose shape can be changed dynamically, thus shifting the phase of light in a controllable, position dependent manner. Using computerized ray tracing, the standard technique for designing and evaluating optical systems, we can simulate both the effect of aberrations and the benefits of using a deformable mirror. The simulations for the extreme case of an oil immersion objective imaging into water (Fig. 2b and c) show that aberrated PSFs computed for a series of depths can be effectively corrected by adjusting the phase for each ray according to its angle using Eq. (2). Spherical aberration can therefore be perfectly corrected by AO. Some aspects of the AO elements should be emphasized. The optical path adjustment needed for typical biological applications would require correction of about 2 micrometres for every 10 micrometres of depth (substituting in Eq. 2 \( n = 1.518 \) (oil) or \( n = 1.333 \) (water) and \( n' = 1.43 \) (cytoplasm) with 65° rays). The number of elements (resolution) in the AO can be small since the correction curve versus angle is smooth and easy to fit by the adaptive mirrors lowest modes of surface displacement.

Fast dynamic focusing without sample perturbation

One goal in developing microscopes for in vivo imaging, is to maximize the rate of collecting three-dimensional image stacks. A new imaging platform developed at UCSF (OMX) can acquire four-dimensional multiwavelength data at rates of more than 10 three-dimensional images a second. A key problem when doing such fast sweeping of the focus is how to avoid distortion of the cover slide and the sample by forces transmitted through the immersion liquid in response to the objective lens motion. Simulations demonstrate that it is possible to add a global curvature term to the AO surface thus shifting the focus with no movement of either the objective or the stage (see Fig. 2a, dashed rays). Since shifting the image plane from the optimally corrected focal plane is associated with increasing aberrations, the respective aberration correction terms must also be added to the surface shape of the AO element. The path length change for simultaneous focusing and correction is (see Fig. 1):

\[
\Delta OP = Dn' \cos \theta'. \quad (3)
\]

Sufficient stroke (maximal axial movement) of the AO will yield a focus shift in the sample space that can cover the thickness of typical biological samples. AO mirrors with a stroke of ±50 micrometre in their lowest mode (mirror phase

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Fig. 1. Calculation of phase aberration for sample depth as a function of angle, \( \theta \). (a) Phase correction for image at depth \( D \). (b) Phase and focusing correction from slide surface into depth \( D \). The phase difference is evaluated from the difference in length between the solid and dashed lines measured from the source to the dotted line representing a plane wavefront.

Equation 2

**sample**  
a

Equation 3

**sample**  
b

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Fig. 2. Correction of Spherical Aberration for an oil immersion objective imaging into a water embedded sample. (a) Schematic optical set-up. The adaptive optical element is inserted in a plane conjugate to the back pupil plane to adjust angle dependent phase aberration. Solid rays: focus under the cover slip; no correction by the AO element is needed. Dashed rays: focus change and phase correction performed by the adaptive element correct the rays paths for a focal plane within the sample. Note that the aperture changes with focus but the effect is very small (exaggerated in the figure) and can be neglected. (b1) Calculated PSF of an ‘ideal’ microscope. (b2) Plotted ray paths emerging from a point under the microscope (bottom fan) and exiting the microscope to focus at the image plane (top fan of rays). (b3) Enlarged view of rays converging onto the focal point. (c1) The PSF computed with a layer of water showing the typical spherical aberration. (c2) The geometrical optics pattern due to refraction in a layer with refractive index different from the immersion oil (depicted in the top ray fan). (c3) Enlarged view of (c2) near the focus, with the geometrical optics pattern corresponding to the aberrated PSF of c1. (b4) The PSF computed for the ideal microscope optics in which the adaptive element introduces the phase corrections according to Eq. (2) for the layer of water. The PSF shows a spherical aberration ‘inverted’ to that in panel c1. (c4) The PSF for imaging into water with phases corrected by the adaptive element, showing recovery of the non-aberrated PSF.

聒 is twice the stroke/wavelength) and setting times of milliseconds are now becoming available (e.g. Mirao, Imagine eyes, Orsay, France). Three-dimensional data collection would therefore incorporate adjustment of the AO element for each section of the three-dimensional data stack to shift the focus while simultaneously correcting the spherical aberration. The speed of present AO elements is faster than objective lens piezo closed loop drives presently applied to fast focusing applications. Note that the range of focus changes (ca. 50 micrometres) is small compared to the focal length of the objective (typically 2–3 mm) and the distance to the input pupil. Therefore, the magnification and numerical aperture changes by this method of focussing are small, and consequently the changes in the collected intensity and the resolution are small.

We have simulated the simultaneous focussing and correction of depth aberration for an oil immersion lens focussing into water using the ray tracing program Zemax (Fig. 3). These simulations model the microscope using real designs for the objective, tube lens and relay lenses (Yamaguchi, 2003, see Tables 1 and 2 for the detailed design of the tube lens and the objective). The adaptive element is modelled as a plane which introduces an ideal position dependent phase delay over the beam diameter. In order to quantify the aberration of the PSF, we use the Strehl ratio as a figure of merit. The Strehl ratio is defined as the ratio between the peak intensity for the image of a point source in the real system and the peak intensity for an ideal system of the same NA (Hardy, 1998). Thus, an ideal diffraction limited optical system has a Strehl ratio of 1.0. In Figure 3 the Strehl ratio is calculated as a function of water depth for both the case of mechanical focussing and of focussing with the AO element. The figure shows perfect correction of the aberrations using an ideal AO element compensating according to Eq. (3) up to a depth of 30 um. It is, in principle, possible to focus deeper (see Section ‘Discussion’). Magnification changes in this range were about 0.1%.

Available adaptive elements have a finite number of actuators and fall into two categories. Shape mirrors have a continuous front surface but are therefore limited in creating large amplitude high spatial frequency patterns. Furthermore, to apply spherical aberration and high-order phase corrections to shape mirrors which can only pull the membrane, a bias must be added to the phase. Segmented mirrors have diffraction effects from the edges of the segments and typically have much lower stroke than the shape mirrors. We have modelled a segmented mirror correcting the depth aberration and compared it to shape mirrors. We find that more actuators are required to achieve the same correction than for a shape mirror.

This configuration of the AO element conjugate to the back pupil plane can also correct the remnant aberrations of the microscope objective itself. These aberrations contain higher
spatial frequencies at the edge of the pupil but smaller phase shifts than are required for focussing. For this application, high-density MEMS AO elements with small strokes could be used (Bifano et al., 2004).

Correction of space-variant sample-induced aberrations

The second configuration (called multiconjugate AO in astronomy, Ragazzoni et al., 2000) tests the possibility of employing a small number of adaptive elements to correct for space-variant aberrations introduced by three-dimensional refractive index fluctuations within the sample. In imaging through live cells, the optical path transverses organelles such as the nucleus and the mitochondria, each with a different refractive index (Ross, 1954; Kam et al., 2001). Three-dimensional Differential Interference Contrast (DIC/Nomarski) microscopy allows one to measure the index of refraction gradients. Then line integration, a rapid computing step, can be used to produce a complete three-dimensional map of the sample refractive index (Van Munster et al., 1997; 1998; Kam, 1998; Kam et al., 2001; Arnison et al., 2004; King & Cogswell, 2004). Line integration by itself requires several milliseconds of computer time. Line integration with deconvolution (LID) of a $512 \times 512$ DIC image requires 0.2 s using a 2.2 GHz Pentium 4 XEON computer. Optimization of the fast fourier transform code can shorten processing time by a factor of 2–5. Since the processing is two-dimensional, simple parallelization using computer clusters will require 0.2 s or less also for three-dimensional stacks. The acquisition of a series of DIC images, the calculation of the three-dimensional refractive index map and the setting of the adaptive elements can thus all be performed very rapidly; therefore this approach is practical for real experimental conditions in live cell imaging.

The mode of correction here requires a different approach from the correction of depth aberration, since the phase shifts measured at the back aperture are position dependent (depend for each wavefront on its point of origin within the sample volume). The way to correct these space-variant aberrations is to guide the wavefront through an ‘inverse sample’ (Fig. 4a) that consists of the opposite refractive index contrasts of the sample. If refraction is negligibly small, an inverse sample would perfectly correct the phase shifts which each ray accumulates in traversing the sample. We show by simulation that this hypothetical solution works very well for the refractive index variations within live cells ($\Delta n \sim 0.06$ between the cytoplasm and the nucleus, Ross, 1954). In practice, the three-dimensional inverse of the sample refractive index can be approximated by a sequence of adaptive elements before the image-forming plane, each compensating for the phase shifts in a finite thickness range within the sample (Fig. 4b). This ‘multiconjugates’ approach in telescopes employs several adaptive optical elements, each correcting aberrations due to atmospheric turbulences at the optically conjugated height. Typically, one height is right above the telescope, and others are several kilometre above. The following analyzes possible implementations of this approach in microscopy.

A single AO element could correct the aberrations perfectly at a single point. Multiple AO elements conjugated to multiple planes in the sample are required in order to correct the aberrations over a finite field of view. To determine the correction applied by each AO element, the optical path along the z-axis can be summed for each position in the plane.
wavefront (see for example, Eq. 3.23 in reference 26) (‘Parallel-sum’). However, in contrast to astronomical telescopes, high-resolution light microscopy deals with large apertures, and the ray paths do not lie along the z-axis. Instead, the projection of a slice of sample volume onto a conjugate plane will be better estimated by a conical fan of rays projected from the origin on the optical axis (‘Fan-sum’). Unfortunately, such conical projection depends on the source point in the imaged field, and the correction deteriorates when moving away from the field center. Tokovinin discusses an optimal representation of a continuous three-dimensional sample by a finite number of adaptive elements for compensation of the aberrations within a finite field of view (Tokovinin et al., 2000; Ragazzoni et al., 2002). In essence, Tokovinin’s approach ‘blurs’ the refractive index over a region that increases with the distance from the conjugate plane to obtain an average contribution for phase shifts of rays falling on the adaptive element from all directions within the field. This approach compromises somewhat the best correction for a small field of view, but improves the overall correction for the imaged field (Fig. 5a). As the number of AO elements increases, the difference between Parallel-sum Fan-sum, Tokovinin and the inverse sample approaches decreases.

The simulation described here includes ray tracing through a three-dimensional volume with random fluctuations in the refractive index, generated by randomly positioned Gaussian

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**Fig. 4.** Schematic optical setup for correcting sample-induced aberrations. Sample inhomogeneities are simulated by ellipses with refractive index higher than the medium. (a) Correction by an ‘inverse sample’. When the refractive contrast between the spheres and the medium is small, refraction effects are small. The ‘inverse sample’ made by the opposite refractive contrast compensates for the sample-induced phase shifts and corrects the aberrations. (b) Schematic presentation of a triple-conjugate corrector applied to approximate the ‘inverse sample’. See text for the approximation algorithms applied in the simulations.
spheres each having refractive index higher by $\Delta n$ above the background, and $M$ adaptive devices each arranged to compensate $1/M$ of the sample thickness. The ray tracing simulations do not take scattering effects into account. We again use the Strehl ratio to quantify the correction over the field of view due to sample-induced aberrations. It is calculated in our simulations by integrating the complex amplitude of the normalized light field over the pupil plane and taking the magnitude squared (Hardy, 1998). The Strehl ratio is a useful characterization of the PSF only for small aberrations, since more complex distortions of the point image take place with large aberrations (Kam et al., 2001). To use the Strehl ratio as a good figure of merit we restrict our simulations here to values of $\Delta n$ that allow comparison of Strehl ratios above 0.1. This simulation allows us to change $\Delta n$ and the size scale because live cells consist of a wide distribution of sizes, shapes and $\Delta n$.

We tested the correction of aberrations for distributions of spheres by a three-dimensional inverse sample (Fig. 5b). As expected, the inverse sample corrects the aberrations perfectly if refraction is negligible. By suppressing refraction in the simulated ray tracing we indeed obtain a Strehl ratio of 1.0. Refraction causes rays to trace different paths in the real and inverse samples, and thus the phase correction will be imperfect if $\Delta n$ is large. With refraction, the inverse medium correction maintained Strehl ratios $>0.85$ throughout the

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Fig. 5. (a) Plot of Strehl ratio versus field position showing multiconjugate correction for sample-induced aberrations. Sample consists of 500 Gaussian spheres with refractive index differences $\Delta n = 0.003$. Open squares, triangles and circles: two conjugates approximated by Parallel-sum, Tokovinin and Fan-sum methods, respectively; Solid squares triangles and circles: four conjugates, Parallel-sum, Tokovinin and Fan-sum methods respectively. (b) Strehl ratio as a function of refractive index contrast for an axial source and 500 randomly positioned Gaussian spheres. Filled circles: uncorrected. Filled squares and triangles: after correction by 'inverse medium' when refraction is suppressed, and with refraction, respectively. Open circles: correction by two conjugates. Filled diamonds: correction by four conjugates.
field for $\Delta n = 0.01$ while the uncorrected Strehl ratio was <0.1. The inverse medium can thus be viewed as the best possible correction, equivalent to a large number of multiconjugates. While there are schemes for writing a three-dimensional refractive index map into a polymer media, such approaches are not realistic for dynamic imaging in live microscopy. We have therefore to resort to methods that utilize only a few conjugate planes. Fig. 5a compares the Strehl ratio for the simulated fluctuating refractive index medium, as a function of source distance from the optical axis, for six cases: correction at either two or four conjugate planes using the Parallel-sum, Fan-sum, and Tokovinin’s corrections. The correction by four conjugates using the Fan-sum method is best on-axis, but degrades as the source moves off-axis. The Tokovinin method does not correct as well on-axis but the level of correction is maintained as the source moves off-axis. We also tested the effect of ‘segmentation’ (or number of actuators) in the adaptive element. For spheres the Strehl ratio approached 1.0 when the effective pixel size related back to the sample became smaller than $\sim 1/3$ of the sphere diameter.

Several details are worth noting. An initial three-dimensional refractive index map is required before fluorescent data is collected. This refractive index map could be updated before every three-dimensional fluorescent data acquisition, or periodically when sample changes are slow and the fluorescence acquisition rate is very fast. Ideally one would want very high resolution of the AO elements, approaching in principle the resolution of the image itself. Achieving good compensation, for nuclei (3–10 $\mu$m size), for example, might be possible using AO conjugates with 50 or more elements (actuators).

**Discussion**

Using ray tracing simulations we have tested two configurations for including AO elements in high-resolution light microscopes to correct aberrations associated with imaging of live thick biological specimens. Using adaptive optics in wide-field microscopy makes possible the correction of parallel data acquisition at different depths and thus is an optimal strategy for collecting live dynamic image information. Because the entire frame is collected at once in wide-field, the response time of the adaptive elements can be much slower compared to the microsecond rates needed for adaptive optics correctors in scanning confocal microscopes (Albert et al., 2000; Booth et al., 2002). We show that correcting for spherical aberration caused by focussing into sample buffer even for the extreme refractive index mismatch with an oil-immersion objective is straightforward, simple to apply, and yields the double benefit of also providing a mechanism to make very rapid focus changes without perturbing the sample. Because the refractive index of live cells is on the average 1.43, and varies between different tissues, an oil immersion lens is actually the most appropriate to use with adaptive optics. When corrected with AO, it also has the benefit of providing the highest possible numerical aperture and hence the best resolution and light gathering power. Furthermore, complete correction of depth dependent numerical aperture allows rapid, space invariant computational deconvolution methods to correct for the remaining out-of-focus blurring.

With large stroke AO devices becoming available, it should be possible to image through many cell layers (100 $\mu$m or more) in intact tissues. For optimal imaging of such very thick tissues it will also be necessary to develop methods to correct for higher order aberrations, as well as for dealing with light scattering. Two-photon microscopy, which gives good performance in highly scattering tissue, will greatly benefit from adaptive optics correction.

Once an AO element is employed to correct depth-dependent aberrations at the back aperture, it would also be possible to simultaneously correct for additional optical aberrations such as remnant phase shifts and asymmetries existing even in the best-selected objectives. These phase shifts increase dramatically towards the high, peripheral acceptance aperture of the objective, which amounts to high order aberration terms (Juskaitis & Wilson, 1997; Beverage et al., 2002; Hanser et al., 2002). They show temperature and “age” dependence, especially serious in heat-incubated environmental chambers used for imaging live biological samples. Such objective-dependent phase aberrations can be determined optically (Juskaitis & Wilson, 1997; Beverage et al., 2002) or computationally (Hanser et al., 2002) but their in situ correction using adaptive elements will be fast and will increase image resolution and contrast. It would be also possible to use the same adaptive optic element to correct for the small residual wavelength dependent aberrations that otherwise would degrade multiwavelength imaging. Finally, if AO elements were to become a standard feature in optical microscopes, it might be possible to greatly simplify the design of the objective lenses (e.g. to get rid of correction collars and to reduce number of lenses required for chromatic correction) and to achieve better overall performance and longer working distances using the flexible dynamic characteristics of AO elements to relax the multiple constraints imposed in objective design.

The second mode explored here, tackles the more challenging problem of correcting the smaller, but more complex, spatially variant aberrations arising from the optical properties of the sample itself. In wide-field imaging, a perfect correction would require the construction of an inverse sample having all the resolution and depth of the real sample, but the opposite change in index of refraction. A more practical alternative is to use a small number of multiconjugate AO elements. To explore the feasibility of this approach, we have simulated the effects of using two or four AO elements with a variety of methods for calculating how each should be set given a three-dimensional map of refractive index variation within the sample. These simulations indicate that useful corrections...
can be obtained using two multiconjugate elements, while using four yields excellent results.

The use of several reflecting adjustable mirrors may be possible, but physically cumbersome, for applications in microscopy. Transmitting adaptive elements (such as liquid crystal spatial phase modulators) capable of introducing position dependent phase shifts through their aperture (Dayton et al., 2002; Lee et al., 2005) can make this multiconjugate approach practical and effective. They have a sufficiently high pixel density for correction of sample-induced aberrations, and their setting speed, being in the millisecond range, is reasonable for wide-field microscopy. However, the present devices have high transmission losses, imposing a serious constraint for their use in tandem. Thus with current devices, it is most reasonable to consider only the two multiconjugate correction scheme.

The introduction of adaptive optics into light microscopy optimize high-resolution imaging under the less than ideal conditions typical in biological applications, increase three-dimensional sharpness and contrast for thick specimens, and allow the detection of finer details and weaker molecular signals inside live cells. As outlined in the introduction, this last point is the critical issue in live cell imaging. An important aim of this paper is the stimulation and the development of new adaptive optics hardware and software in wide-field microscopy.

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