Correcting Depth Aberrations with Remote Focusing using Adaptive Optics in High-Resolution Wide-Field Microscopy

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ABSTRACT

Depth aberrations are a major source of image degradation in three-dimensional microscopy, causing a significant loss of resolution and intensity deep into the sample. These aberrations occur because of an inevitable mismatch between the sample refractive index and the immersion medium index. We have built a wide-field fluorescence microscope that incorporates a large-throw deformable mirror to correct for depth aberrations in 3D imaging. We demonstrate a corrected point spread function imaging beads in water with an oil immersion lens and a twofold improvement in peak signal intensity. We apply this new microscope to imaging biological samples, and show sharper images and improved deconvolution.

Keywords: Adaptive Optics, Microscopy, Biomedical Imaging

1. INTRODUCTION

In modern three-dimensional fluorescence microscopy, maintaining the highest resolution and signal intensity is critical for studying sub-cellular structure and dynamics. Unfortunately, high numerical aperture objectives only give perfectly corrected images just below the coverslip. As the focal plane is moved deeper into the sample, the image degrades due to the difference in path length caused by the refractive index of the sample replacing that of the immersion media. This degradation, the depth dependent aberration, can quickly become serious. Imaging 20 microns into a live sample (index \( \approx 1.36 \)) with an oil immersion lens, the peak intensity of the point spread function (PSF) will drop by 3x and the width of the PSF in the axial direction will increase by 2x.

Furthermore, the degradation of the PSF limits the power of deconvolution to improve the image contrast by re-assigning out-of-focus light to its point of emission\textsuperscript{2}. Deconvolution requires an accurate knowledge of the PSF. If the PSF is highly aberrated, then the blurring cannot be deconvolved with the unaberrated PSF measured at the coverslip. Deconvolution with a depth dependent PSF has been implemented\textsuperscript{3, 4}, but this technique is computationally intensive, and does not improve the signal to noise ratio by restoring the peak intensity of the original signal. This is in general true of the many sophisticated post-acquisition image processing algorithms that are available.

The problem of depth aberration can be solved by matching the sample index and the index of the immersion medium, but this is frequently not feasible or desirable. For example, the index of fixed cells can be matched to that of immersion oil\textsuperscript{5}, but this option is not available for live imaging. A water immersion objective can be used to more closely match the index of a live sample; but this solution merely pushes the point at which aberrations become serious from a few microns to a few tens of microns, and there are many cases when one would like to image hundreds of microns into live tissue\textsuperscript{6}. Furthermore, water lenses must be carefully adjusted to correct for the coverslip thickness or they will suffer from the same types of image degradation that they are meant to correct.

Recently we proposed correcting the depth aberrations by correcting the phase of the emitted light in the back pupil plane of the microscope\textsuperscript{7}. The depth aberrations can be corrected in the back pupil plane across the entire field for a given depth because the path differences of the emitted light that give rise to the depth aberrations are only dependent on the angle of the emitted light and not on field position. Here we implement this proposal for the correction of depth

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Aberrations with a deformable mirror (DM) conjugate to the back pupil plane in both the excitation and emission paths of a wide-field fluorescent microscope. This is the first use of adaptive optics in three-dimensional wide-field microscopy.

Adaptive optics has been implemented in confocal microscopy to correct aberrations, both depth aberrations and specimen-induced aberrations, on a point-by-point basis. An important drawback to the schemes that have been proposed so far is that they require several images to be taken to optimize the aberration correction. This presents a serious problem for live imaging in biology because the fluorescence intensities can be weak and susceptible to rapid bleaching.

Another benefit to controlling the phase in the back pupil plane of the microscope is that it allows for remote focusing. As discussed below, the phase can be corrected to compensate not only for the refractive index mismatch, but for the change in focal plane as well. This can be important for rapid three-dimensional imaging; moving the sample rapidly can cause vibrations due to coupling between the objective and sample through the immersion liquid.

2. RESULTS

The approach we follow is to correct the depth aberrations which are the result of the ray path length differences illustrated in figure 1. Fig. 1(a) illustrates the path length difference between a ray from a point source a depth d below the coverslip in a material with index \( n_1 < n_2 \) (solid line) and a ray from a point source in a material with \( n_2 = n_1 \) (dashed line). Because the path length difference is only a function of ray angle and not field position, the path length difference can be corrected for the entire field in the back pupil plane of the objective. For an objective that obeys the sine condition, the phase correction that must be applied in the back pupil plane is

\[
\phi = 2\pi \frac{(NA)d}{\lambda} \left( \sqrt{1 - \left( \frac{(NA)\rho}{n_2} \right)^2} - \sqrt{1 - \left( \frac{(NA)\rho}{n_1} \right)^2} \right)
\]

where \( \rho \) is the normalized radial coordinate in the back pupil plane, \( \lambda \) is the wavelength of the emitted light, and NA is the numerical aperture. Figure 1(b) shows the path length difference between a point at the coverslip and a point at a depth d below the coverslip. Correcting for this path length difference in the back pupil plane, the sample does not have to be moved so this accomplishes focusing with depth correction. In this case the correction is

\[
\phi = 2\pi \frac{(NA)d}{\lambda} \left( \sqrt{1 - \left( \frac{(NA)\rho}{n_2} \right)^2} \right)
\]

We have applied the correction given by equation 1 to a deformable mirror conjugate to the back pupil plane of the mirror (See Methods). Figure 2 shows an image of a 200nm Yellow-Green bead at the coverslip. Because the bead diameter is below the microscope resolution, the image of the bead is a measurement of the point spread function (PSF) of the microscope. As can be seen from the figure, the PSF is well corrected with a full-width half maximum (FWHM) of 270nm which is about 35% larger than the theoretical FWHM of 202nm for 520nm emission light with a numerical aperture (NA) of 1.285. This is comparable to the PSF measured with a silver-coated flat mirror in place of the deformable mirror. The FWHM of the PSF in the axial direction is 700nm. The most significant feature of the in focus PSF is the grid of spots around the central peak; each of these small peaks is about 1% of the maximum intensity of the central spot. This feature is due to the small non-uniformity of the deformable mirror at each actuator position (commonly referred to as print-through) which diffracts light away from the central peak into spots a distance \( f_1(\lambda/\Lambda)/M_{\text{sph}}=1.2\mu\text{m} \) (in sample coordinates) away from the center of the PSF, where \( \Lambda \) is the actuator spacing. A deformable mirror with more actuators would produce spots farther away from the central peak. The print-through also has a significant effect on the out-of-focus light in the PSF. As can be seen in fig. 2(c) and 2(d), the out-of-focus blur takes on a square shape due to the square actuator array. The shape of the PSF is different above and below the focal plane because the print-through is not symmetric in the axial direction. Far away from focus, because of Fraunhofer diffraction, the PSF starts to resemble an image of the print-through. The effect of print-through is an important difference between the use of adaptive optics in wide-field imaging and in confocal and two-photon imaging where the pinhole or the nonlinearity effectively removes all but the central peak of the PSF. We have used phase-retrieval to calculate the wavefront in the back-pupil plane from the PSF. The deformable mirror print-through is clearly visible in the back pupil plane with a magnitude of roughly 30nm.
Figure 3 shows images of a 200nm bead 67um deep in a glycerol/water mixture with index 1.42. First images were taken without correcting for the depth aberration – the deformable mirror was flat. Then images were taken with the shape of the deformable mirror set by equation (1). Because the uncorrected image was taken first, any bleaching of the sample (estimated to be about 4% per image stack) would result in a higher intensity for the uncorrected image. As is clear from figures 3 and 4, the correction achieves several important results. First, the peak intensity of the corrected image is a factor of two larger than the uncorrected image. Second, the correction removes the low intensity “pedestal” of light around the main peak in the uncorrected image as can be clearly seen in the logarithmic scale image, fig. 3(c). This is largely removed by the correction; this energy has been returned to the central peak. Third, the shape of the PSF (compare figs. 3(e) and (f)) after correction is almost exactly the same as that of the PSF at the coverslip which should result in much better deconvolution results. Lastly, fig. 4b, the width of the peak in the axial direction is significantly reduced after correction resulting in a higher axial resolution. The lateral plane full-width at half-maximum of the peak is not significantly changed by the correction as seen in fig. 4a. This is because the depth aberrations do not increase the width of the central peak very much. The aberrations spread the energy removed from the central peak out broadly.

Of course, the goal of using adaptive optics in fluorescence microscopy is to correct images of biological samples. Figure 5 shows lateral images 24um below the coverslip of UMUC bladder cancer cells with GFP-TRF1 labeled telomeres; fig. 5(a) shows the uncorrected image and 5(b) shows the image with the depth aberration corrected by adaptive optics. The autofluorescence of the cell and nucleus is unchanged by the correction of depth aberrations, but the GFP-TRF1 signal is brighter by a factor of two over the background. Because adaptive optics corrects the PSF throughout the sample, it greatly improves the ability of deconvolution algorithms to remove the blurring of the object caused by the PSF. This is demonstrated in figure 6 which shows the deconvolution of images of actin labeled with Alexa488-Phalloidin in B16F10 cells. The deconvolved image (b) of the AO-corrected data shows significantly less background than the deconvolved image of the uncorrected data. These xy sections are 4.4um below the coverslip. Interestingly, the difference in the raw images is not as great.

In addition to correcting the depth aberration, the deformable mirror also has the capability to focus into the sample as described by equation (2). Figure 7 is an example of using the deformable mirror to focus through a fixed C. elegans sample. The images in fig 7(a) are slices through a three-dimensional data stack taken using the deformable mirror to focus in z, and are comparable to the images taken in fig 7(b) using mechanical focusing. The remote focusing configuration is very sensitive to aberrations in intermediate image planes because the beam diameter changes as a function of depth. Thus, the improvement due to depth correction may be negated by aberrations that are uncorrected as the system is focused into the sample.

3. DISCUSSION

Correcting depth aberrations with a deformable mirror improves both the peak intensities and the deconvolution of images taken below the coverslip by removing the depth aberration contribution from the PSF. This allows the use of fast space-invariant deconvolution algorithms instead of depth-dependent algorithms. This is significant because it improves both the signal to noise ratio and the resolution in biological imaging where photons are in short supply. But the deformable mirror provides several challenges to three-dimensional imaging and the performance still does not achieve what is theoretically possible.

While the correction of a bead in glycerol, figs. 3 and 4, shows an impressive improvement in intensity due to the correction, the factor of two improvement is still much less than the factor of four that should be possible based on the theoretical decrease in intensity predicted by the depth, NA and index of refraction mismatch. There are two important factors that contribute to this effect. The first is the affect of residual aberrations on the change in intensity due to depth aberration without depth correction. Residual aberrations will cause a decrease in the maximum intensity at the coverslip, but this intensity decrease does not add to the intensity decrease due to depth aberrations, so aberrations lead to a smaller intensity loss with depth. The second factor is the ability of the mirror to conform to the shape given by equation (1). The residual error of the mirror shape increases with depth so that as the imaging plane goes deeper and the possibility for improvement becomes greater, the improvement in Strehl ratio decreases. Addressing the first problem, to improve the performance of the system, we must reduce the residual aberrations in the system as much as possible. There are several important possible sources of aberration in the system. The first is the optics in the system itself. The objective and tube lens will not be perfect and the performance of objectives in particular has been known to change over time. The mirrors in the system will also be an important source of aberrations. Most silver mirrors are specified to
have an root-mean square surface flatness of $\lambda/10$. According to the Marechal criterion\textsuperscript{16}, this will result in a Strehl ratio of roughly 0.6. Thus having a few mirrors in the system can be a significant source of aberration.

Two other important factors that will affect the performance in biological samples are the refractive index variations of the sample itself (sample-induced aberrations) and scattering. Similar to the residual aberrations in the optical train, sample-induced aberrations can affect the performance of the system in a nonlinear way and will affect the measured performance of the microscope in correcting depth aberrations. Scattering will also affect the ability of the microscope to recover the intensity lost to depth aberrations since scattering will disperse the light in a random way so that it cannot be imaged.

Another important feature of three-dimensional wide-field microscopy is the three-dimensional nature of the PSF. As can be seen in figures 2 and 3, the point-spread function of the system contains many weak features outside the central peak. Most notable is the grid of points due to the deformable mirror print-through. In confocal microscopy, only light in the central peak is accepted by the pinhole, so the extra structure caused by the print-through only leads to a small loss of intensity. In wide field microscopy, the object (or fluorescence emission) is convolved with the full 3D PSF, so that the structure in the PSF can be visible in the image. Deconvolution is important for removing this structure, and the AIDA deconvolution program works reasonably well in removing this structure. Myopic deconvolution, allowing the PSF to vary under a harmonic constraint along with the object to optimize the reconstruction, is an option in the AIDA program that we use, and it would be interesting to tailor the PSF constraint for the case of adaptive optics where the spatial frequency of the print-through is known, but the exact structure could be optimized to account for the details of the PSF.

There are many modifications to the microscope design that we present here that could improve correction of depth aberrations and extend the design to the correction of sample-induced aberrations. A deformable mirror with more actuators could be used to more faithfully approximate the depth correction term given by equation (2). One difficulty with this is that the high-actuator devices typically can shift the phase by at most a few wavelengths, so using only a high-actuator mirror would severely limit the depth correction. To get around this difficulty, a microscope with two deformable mirrors in a “woofer-tweeter” configuration could be designed using one low-actuator large-throw mirror and one high-actuator low throw mirror. Lastly, the ultimate goal of applying adaptive optics in microscopy is to correct all aberrations including those introduced by the refractive index variations of the sample itself. Thus, inserting additional deformable mirrors conjugate to planes in the sample itself for correcting sample-induced variations over the field of view is an important future goal.

4. MATERIALS AND METHODS

4.1 Microscope Design

Figure 8 shows the optical layout of the microscope. The objective (Olympus, 60x, 1.42 PlanApo N) collimates the light from the object. The tube lens and lens $f_1$ (350mm achromat, OptoSigma) create an image of the objective back pupil plane on the deformable mirror. The magnification is chosen so that the desired NA of the system fills the deformable mirror. Lens $f_2$ (1500mm singlet, Newport Corp., or 800mm doublet, OptoSigma) then creates an image on the CCD camera. The total magnification of the system, $M_{obj}(f_2/f_1)$ is chosen so that the object is sampled at least the Nyquist frequency on the CCD camera. Here, $d_{pixel} = 50.6$ nm or 94.6nm in sample coordinates. We oversample so that we accurately measure the PSF without having to interpolate the data. The CCD camera is from Astronomical Research Cameras, Inc. and uses a cooled CCD57-10 chip with a 13um pixel size from e2v Technologies.

The excitation light, 488nm from a Coherent Innova laser, enters the system through a multimode fiber with a 600um core (Ocean Optics). The end of the fiber is imaged onto the deformable mirror with lenses $f_{ex1}$ and $f_{ex2}$ and inserted into the main optical path with dichroic 1 (Semrock FF495-Di02). Thus, the excitation is also corrected for the depth aberrations. While this is not critical for wide-field illumination, correction of the excitation is equally as important as correction of the emission in techniques such as structured illumination microscopy\textsuperscript{17}.

The final optical path in the system injects a 632nm HeNe laser into the system to monitor the shape of the deformable mirror. The beam from a HeNe laser is cleaned and expanded with a pinhole and then collimated. The light is inserted into the optical path with dichroic 2 (Semrock FF579/644-Di01). It then follows the optical path until it is taken out of the main optical path with dichroic 3 (Semrock FF579/644-Di01) and sent to a Shack-Hartmann wavefront sensor (Imagine Optic Haso 32). Lenses $f_{sh1}$ and $f_{sh2}$ demagnify the beam to image the deformable mirror onto the lenslet array.
4.2 Deformable Mirror Control

For the deformable mirror, we chose the Mirao52D from Imagine-Optic\textsuperscript{18} because it is capable of large displacements and thus will permit the correction of aberrations deep into a sample. The mirror has 52 actuators on a square grid with a 2.5mm spacing and 15mm diameter. The mirror is capable of a maximum displacement of +/-75 microns for the focus ($Z_2^0$) mode and +/-8 microns for first order spherical aberration ($Z_4^0$). The mirror can take the shape of any Zernike mode through order 4 with an rms wavefront error of less than 20nm. Because the mirror cannot set the higher order terms, the Strehl ratio will degrade with depth, as the amount of the higher order spherical terms needed for correction increases. Thus, the depth of correction will be limited by the residual aberrations and not the maximum displacement of the focus mode.

We control the mirror by measuring the wavefront of the HeNe laser with the wavefront sensor (reference path in figure 2). We reference the wavefront to a measurement with all actuators set to zero, and then measure the wavefront for each actuator individually turned on yielding a 1024x52 matrix since we measure the wavefront on a 32x32 array. To set a desired mirror shape, we use the standard singular value decomposition technique\textsuperscript{19} to determine the matrix $S$ which will yield the actuator values for a desired wavefront. Typically, we only retain the first 45 singular values.

4.3 Sample Preparation

To measure the PSF, we imaged 200nm diameter Yellow-Green fluorescent beads (Molecular Probes F-8811). Because the beads are smaller than the diffraction spot, its image represents the PSF of the microscope. The beads are dispersed in water at a concentration of 2\% by weight and were diluted by an additional factor of 10\textsuperscript{6} in water to a concentration of 3.9x10\textsuperscript{6} beads/ml\textsuperscript{20}. To image beads at 0 depth (on the coverslip), 15ul of bead solution were dried on a coverslip and mounted on a slide with 5ul of glycerol. To image beads below the coverslip, #2 coverslips were glued to a slide on either side of a #1.5 coverslip using optical adhesive (Norland 61). The #2 coverslips serve as a support. 200nm beads were dried on the center coverslip and on a 40x20mm #1.5 coverslip. 25ul of a water/glycerol mixture was put on the slide and the larger coverslip was placed on top and sealed with nail polish. The relative concentration of water and glycerol in the mixture was adjusted to yield an index of refraction of 1.42 for the mounting solution\textsuperscript{21}. The distance between the top coverslip and the slide varied between 45 and 75um.

B16F10 mouse cells were imaged. The cells were grown on Fibronectin coated #1.5 coverslips and were stained with alexa488-phalloidin to label the actin following the protocol developed by Cramer and Desai\textsuperscript{22}. The coverslips were then mounted on slides with PBS buffer and sealed with nail polish. Autofluorescence from C. elegans adult worms was also imaged. Live worms were mounted on agar in M9 buffer containing 1mM Levamisole to paralyze the worms. A #1.5 coverslip was placed over the sample and sealed with nail polish.

4.4 Deconvolution

The deconvolution was performed using the AIDA software package\textsuperscript{23}. We used the myopic deconvolution option in AIDA, allowing the PSF to vary to optimize the reconstructed object, and the initial guess was a Wiener-filtered version of the image. The deconvolutions took approximately 30 minutes each on a Pentium III. The optimized PSF was not significantly different than the original PSF.

5. CONCLUSION

We have designed and built a wide-field fluorescent microscope incorporating a deformable mirror, capable of both correcting depth aberrations and remotely focusing through a biological sample. We have demonstrated that this microscope can correct depth aberrations when imaging below the coverslip. We see improved intensities from fluorescent beads and improved deconvolution from biological images.

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Figure 1: (a) diagram showing the path length difference between an unrefracted ray (dashed line) from a point a depth $d$ below the coverslip and a ray that is refracted at the interface (solid line). (b) shows the path length difference between a ray from a point just below the coverslip (dashed line) and a ray a depth $d$ below the interface.

Figure 2: Measured point spread function of the microscope measured at the coverslip. (a) PSF in focus scaled to maximum intensity. The images are 24um by 24um. (b) PSF in focus, logarithmic scale. A series of faint peaks are visible around the central PSF due to diffraction from the mirror print-through. (c) PSF 6um out of focus away from the coverslip. (d) PSF 6um out of focus towards the coverslip. (e) xz cross-section of the PSF on a logarithmic scale. The image is 24um by 10um.
Figure 3: Images of a 200nm bead in 67um of a water/glycerol mixture with n=1.42. (a) uncorrected image of in focus plane (b) corrected image of in-focus plane: same scale as (a). (c) and (d) are the same as (a) and (b) respectively but on a base 10 logarithmic scale. (e) and (f) are cross-sections through the focal plane on a linear scale.

Figure 4: Plots of the intensity through the center of the 200nm bead along a lateral and the longitudinal axis. The solid line is the intensity of the corrected image, and the dashed line is the intensity of the uncorrected image. This is the same data as figure 3.
Figure 5: Images of GFP-TRF1 labeled telomeres in UMUC bladder cancer cells. (a) uncorrected images 24um below the coverslip (b) depth aberration corrected 24um below the coverslip. The inset is a profile through a GFP-TRF1 labeled telomere along the axial direction.

Figure 6: Deconvolved images of alexa488-phalloidin labeled B16F10 mouse cells. Images are 4.4um below the coverslip. (a) uncorrected image. (b) image corrected by adaptive optics.
Figure 7: Comparison of images taken by focusing with the deformable mirror (a) and mechanical focusing (b). Images are of C. elegans autofluorescence. The top images are taken 6um below the coverslip and the bottom images are xz cross-sections. Images (a) are weaker because this image stack was taken second and the sample exhibited strong photobleaching.
Figure 8: Microscope Layout.