Optical Imaging in Biology and Medicine
Master in Photonics & Europhotonics Master Program

Adaptive Optics for Microscopy

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Abstract

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Contents

1 Introduction

actual techniques in the second part, methods

[3]

The performance of these microscopes is often compromised by aberrations that lead to a reduction image resolution and contrast.

These aberrations may arise from imperfections in the optical system or may be introduced by the physical properties of the specimen.

The problems caused by aberrations can be overcome using adaptive optics, whereby aberrations are corrected using a dynamic element, such as a deformable mirror.

This technology was originally conceived for the compensation of the aberrating effects of the atmosphere and was first developed for military and astronomical telescopes.

Adaptive optics systems have also been introduced for other applications such as laser beam shaping, optical communications, data storage, ophthalmology and microscopy.

[2]

Optical microscopes have long been essential tools in many scientific disciplines, particularly the biological and medical sciences. Conventional widefield microscopes—encompassing transmission, phase contrast and fluorescence imaging modes—are the workhorses of many laboratories. Over the last 25 years, researchers have also made significant developments in 3-D imaging using scanning laser microscopes. This progress started with the confocal microscope, which provides 3-D resolution by using a pinhole to exclude out-of-focus light. Rather than produce a whole image simultaneously, these microscopes scan a laser spot through the specimen, building the image point-by-point. This achievement was followed by several other laser-scanning methods, including the commonly used twophoton fluorescence microscope. Rather than using a pinhole to generate 3-D discrimination, this microscope relies on the nonlinear process of two-photon excitation to ensure that fluorescence is only generated in the focus, where the laser intensity is highest. Various advances in this field have led to improvements in resolution and contrast. Standard laboratory microscopes now regularly produce images revealing 3-D structure on the submicrometer scale. Several new methods of nanoscopy that combine optical and photophysical phenomena can even beat the diffraction limit to resolve details on the tens-of-nanometers scale.

These methods all rely on careful engineering to ensure that the optics operate at the diffraction limit, so that optimum resolution and efficiency are achieved. However, one part of the optical system—the specimen—lies outside the design specification. It is optically inhomogeneous and exhibits spatially varying refractive indices. Hence, the light focused into the specimen suffers from wavefront distortions— or phase aberrations—that degrade the resolution and imaging efficiency of the microscope. The aberrations vary from one specimen to another, so they cannot be corrected by a fixed optical design. Dynamic correction is necessary. This is where adaptive optics (AO) comes into play.

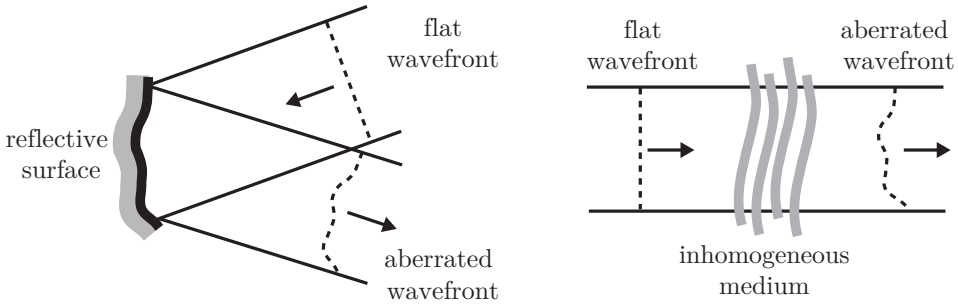
Adaptive optics was originally conceived for use in astronomical telescopes. These AO systems detect aberrations introduced by the atmosphere and use a deformable mirror to remove the aberrations before the light reaches the imaging detector. For imaging systems with small apertures, such as our eyes, the turbulence causes twinkling; for wider telescope apertures, it leads to severe image blurring that limits the resolution of the telescope.

The AO approach has been widely applied in astronomy, and it has also found application in ophthalmic imaging, laser-based fabrication, optical communications and, of course, microscopy. The adoption of AO for microscopes has brought new challenges that have required innovative solutions.

[3]

2 Aberration Measurement and Correction

For the purpose of understanding the operation of an adaptive optical system, it is best to think of aberrations in terms of distortions of an optical wavefront.



(a) Reflection. (b) Transmission.

Figure 1: Wavefront aberrations due to (a) reflection from a non planar surface and (b) caused by propagation through a non-uniform refractive index distribution. Image after [10].

Representing aberrations in this way can simplify the design, control and characterisation of adaptive optics. The choice of modes for a particular application is often influenced by some aspect of the system, such as the deformation modes of a deformable mirror or the statistics of the induced aberrations. Otherwise, the modal representation may be chosen through mathematical convenience. For example, Zernike polynomials are often used for systems with circular apertures as they form a complete, orthogonal set of functions defined over a unit circle

As with all optical systems, microscopes can also suffer from aberrations due to imperfections in the optical components. In practice, no system can be totally free from aberrations and so systems are designed to maintain aberrations below a particular tolerance for a given set of imaging conditions, such as wavelength, magnification and field of view. Significant aberrations can be introduced if a microscope is used outside its design specifications, for example at the incorrect wavelength or at a difierent temperature (see Chapter 11 of Ref. 9).

[2]

2.1 Wavefront Sensing

2.1.1 Interferometry

2.1.2 Shack-Hartman Wavefront Sensor

2.1.3 Indirect Wavefront Sensing

2.2 Aberration Correction

computational

2.3 Deformable Mirror

2.3.1 Liquid Crystal Spatial Light Modulators

2.4 Control Strategies

3 Adaptive Optics Methods applied in Microscopy

AO has been demonstrated in a range of microscope modalities, including conventional widefield microscopes as well as laser scanning systems. The most common implementations have involved confocal and two-photon fluorescence microscopy, both of which are widely used methods in biomedical investigations. Due to aberrations, these microscopes suffer from a significant drop in signal and resolution as the focus is moved deeper into the specimen.

Various research groups have combined these microscopes with direct wavefront sensing and sensorless AO, normally using deformable mirrors for aberration compensation. Ji et al. developed another approach that uses an SLM to implement a pupil segmentation phasing method in a two-photon microscope. AO has also been applied to microscopes using more exotic contrast mechanisms based upon nonlinear optical processes, such as second- and third-harmonic generation or coherent anti-Stokes Raman scattering. Using these various methods, researchers have demonstrated image improvement at depths of up to 100 ìm in mouse embryos and over 200 ìm in brain tissue.

Adaptive microscopy is also finding a role in the imaging of live specimens. It can help to reduce the time required for image acquisition by increasing signal generation and collection efficiency. This is particularly useful in microscopes that rely on nonlinear contrast mechanisms, where any drop in focal intensity has a compounded effect on signal level.

AO can help by allowing the designer to relax the aberration tolerance from the usual fraction of a wavelength up to several wavelengths. This permits a significant reduction in the complexity of the optical system. In configurations where the optical fidelity has been compromised, the AO can be used to correct the residual system aberration, restoring diffractionlimited operation.

We can conclude: specimen induced aberrations lead to reduced signal levels and a deterioration in image quality in optical microscopy, especially in CFM and TPM. For the first time, specimen induced aberrations that occur with various biological specimens have been classified and quantified for the most relevant condition of high NA. The above approach can provide detailed information about the variation of each Zernike coefficient across the scan. As expected from theory, lower NA systems are less susceptible to aberrations than high NA system under otherwise similar conditions. Low order correction would still provide benefits, even though the initial aberrations are smaller. The results presented here quantify the benefit of adaptive optics for biological microscopy and provide the bounds within which these systems must operate. [16]

3.1 Widefield Microscopy

In conventional microscopes, widefield illumination is provided using either transmission optics or, in the case of re ection or uorescence modes, via the objective lens in an epi configuration. In either case, the image quality depends only on the optics of the detection path and is independent of the fidelity of the illumination path. Aberration correction is therefore only necessary in the detection path and a single pass adaptive optics system will suffice. [10]

3.1.1 Structured Illumination Microscopy

[6]

Optical sectioning microscopy is widely used to provide three-dimensional fluorescence images of biological specimens. A common way of obtaining this sectioning ability is through point scanning methods such as confocal or multiphoton microscopy [1, 2]. An alternative is to use a wide-field technique such as structured illumination (SI) microscopy, which retains the sectioning ability of confocal microscopy, but can be implemented in a conventional microscope using an incoherent light source, and without the need for scanning.

In this technique, the image of a grid is projected on the specimen so as to produce a one-dimensional sinusoidal excitation pattern in the focal plane of the objective lens. The resulting fluorescence image, consisting of both in- focus and out-of-focus fluorescence emission, is acquired by a camera. Several images are taken, each corresponding to a different grid position. As the grid pattern appears only in

the focal plane, it is possible to extract an optical section from the spatially modulated component of the images via a simple calculation. [12].

In this paper we describe wavefront sensorless adaptive optics implemented in a SI microscope. It is shown that the final image quality depends predominantly on the imaging efficiency of the illumination pattern's spatial frequency. This imaging efficiency is affected much more by some aberration modes than by others. Consequently, different aberration modes can have significantly different effects on the final sectioned image.

The SI microscope relies upon the projection of a physical grid pattern into the focal plane of the specimen. We assume that the grid object is a sinusoidal transmission mask

those aberrations that affect the grid frequency have the most significant effect on the SI microscope. It is therefore useful to separate aberration modes into two groups: those that affect the grid frequency (referred to hereon as "grid modes") and those that have no influence on this frequency ("non-grid modes"). It is clear that grid modes have a significant influence on the intensity of the sectioned image, whereas non-grid modes have comparatively little effect. The non-grid modes do however affect the resolution.

The specification of a modal aberration correction scheme requires the choice of three components: the aberration representation (the mathematical functions used to describe the aberrations), the optimisation metric (a quantity representing the image quality) and the estimator (the algorithm for estimating the correction aberration).

We would like to choose a metric function M whose maximum corresponds to the highest quality image. find an aberration expansion for which the modes act independently on the metric. This would allow the independent optimisation of each mode. When M is expressed in this form, it is clear that independent maximisation with respect to each xi is possible. Furthermore, as the function is quadratic, the maximum can be found directly from three measurements of M corresponding to three different values of xi - here they reference to [5] so maybe I should put the two together, or see this one as a more advandced version of the previous

$$M = M_0 - \sum_i x_i^2 \tag{3.1}$$

In practice, these measurements would be taken with three different trial aberrations introduced by the correction element. However, M only takes the form shown in Eq. (3.1) if the aberration representation is appropriately chosen.

for each aberration mode, the metric M was measured when adding a given amount of the considered mode, then again when substracting the same amount. Along with the measured value of M when no aberration was added, this allowed us to estimate the initial aberration present in each of the assessed modes and hence to correct these aberrations

Here we want to emphasise that the use of the appropriate metric and aberration modes means that the correction of N modes was performed using only 2N+1 measurements, thereby minimizing the increase in illumination time of the sample required for the correction.

The acquisition time per sectioned image was 300-500ms and the total time required for correction of 11 modes was 7-12s, depending on the sample.

However, M only takes the form shown in Eq. (10) if the aberration representation is appropriately chosen.

We are therefore led to the conclusion that the SI microscope, in comparison to other sectioning microscopes, is more susceptible to certain aberrations (grid modes) and more resilient to others (non-grid modes).

Whilst the SI microscope relies upon a relatively simple optical principle, the image formation process has a complex mathematical description. Similarly, the derivation of a modelbased, sensorless, adaptive optical scheme is a complex process. However, our results show that the scheme is effective in correcting specimen-induced and system aberrations and restoring image quality. For the samples presented here, we found that the aberration mainly consisted of astigmatism, coma and spherical aberration modes.

The adaptive scheme described here has significant advantages over model-free algorithms in that the

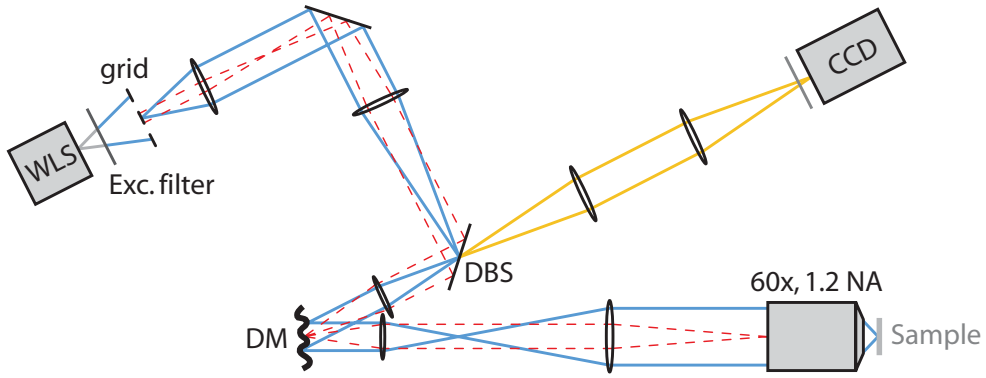
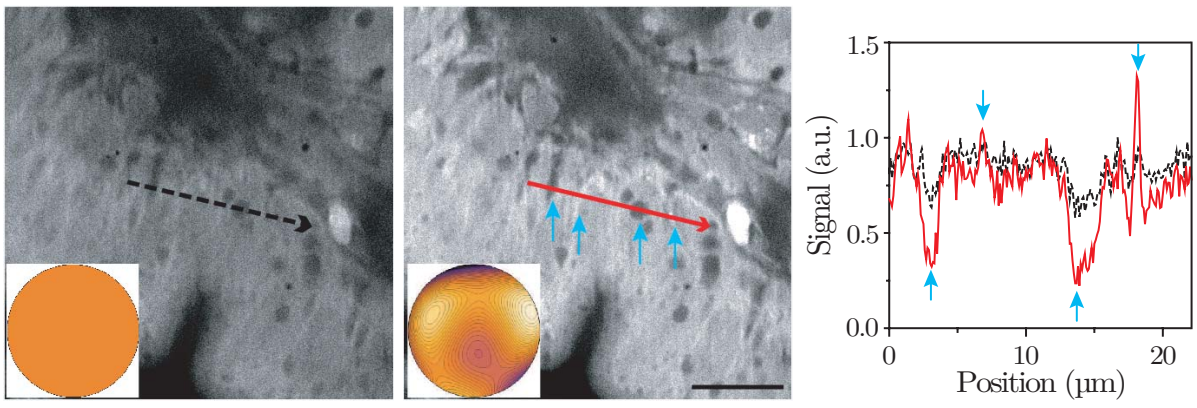


Figure 2: Experimental setup for structured illumination microscopy with aberration correction. WLS - white light source, DM - deformable mirror, DBS - dichroic beamsplitter. The blue rays mark the illumination path; the detection path is shown in yellow. Image after [6].

aberration correction can be estimated using a small number of measurements (2N+1 for N aberration modes). Moreover, as the scheme is mostly independent of the object structure, the appropriate modes have only to be determined once and the same scheme can be used for any specimen.We have also shown that aberration correction can be effectively combined with background subtraction to further improve SI microscope images. In the results presented here, aberration correction was performed as an average over an image frame and therefore would not correct for any local variations in aberrations. If these variations were found to be significant, the image could be formed from several sub-images for which independent aberration correction would be performed.

We have presented a general method that provides an optimal aberration expansion for a chosen optimisation metric. This relied upon the derivation of an inner product from a mathematical model of the imaging process, followed by an orthogonalisation process applied to a set of basis functions, such as the Zernike functions. This process reveals a wealth of information about the effects of different aberration modes on an imaging system – for the SI microscope, it enabled us to derive the sets of grid modes and non-grid modes. **This method could equally be applied to any sectioning microscope to derive aberration expansions that are best suited to that application.**



(a) Uncorrected. (b) Corrected. (c) Line Scan.

Figure 3: Aberration correction in structured illumination microscopy. A fluorescent mouse intestine sample was imaged (a) without (b) with aberration correction with inserts showing the phase induced by the deformable mirror. (c) Profile along the lines drawn on the images, both profiles normalized so that their mean value is identical. As a result of the resolution improvement, the contrast of small sample features (blue arrows) are better defined after (red solid line) rather than before (black dotted line) correction. The imaging depth was approximately 10 µ, sacle bar size 10 µ. Image after [6].

3.1.2 Fluorescence Microscopy

next section, all from [9] uses a standard widefield flourescene microscope but use AOM to correct for spherical aberration due to depth -> no specimen induce correction uses deconvolution to get out of foucs photons corrected

In this paper, we concentrate on the depth dependent aberration which can quickly become serious. Imaging 20 mum a live sample (index of refraction 1.36) with an oil immersion lens causes the peak intensity of the point spread function (PSF) to drop 3-fold and the width of the PSF in the axial direction to increase by 2-folds. [9]

Because wide-field microscopy captures as efficiently as possible every emitted photon ultimately minimizing the sample excitation dose, it is well suited to in vivo imaging in sampleswhere scattering is not too large. Although the out-of-focus photons are in thewrong place, they can be effectively re-assigned to the location of emission by constrained deconvolution algorithms [17]

The problem of depth aberrations 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 the immersion oil, but this option is not available for live imaging.

An important drawback to most 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.

The approach we follow is to correct the depth aberrations withanopen-loop predictive algorithm similar totheapproach taken by Potsaid et al. in correcting off-axis aberrations. This is possible because the depth aberration can be calculated for a given depth into the sample. The depth aberration is the result of depth-dependent path length differences.

Correcting depth aberrations with a DM improves both the peak intensities and the deconvolution of images taken below the cover slip by removing the depth aberration. 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. Unfortunately, the performance does not yet achieve what is theoretically possible.

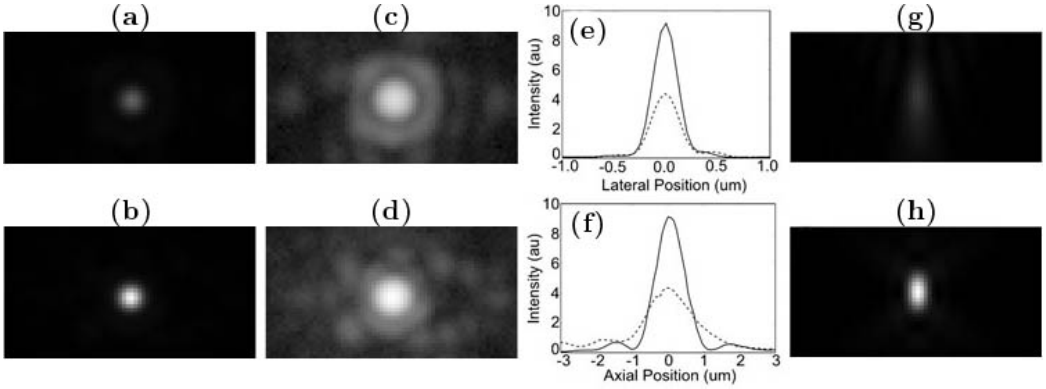


Figure 4: Images of a 200 nm bead 67 µm below the cover slip in a water/glycerol mixture with n = 1.42. (a) Uncorrected image of in-focus plane. (b) Corrected image of in-focus plane. Images (c) and (d) are the same as (a) and (b), respectively, but on a logarithmic scale for better visualization. (e) and (f) are line profiles of the intensity through the center of the bead along the lateral and the longitudinal axis, respectively. The dashed line is from the uncorrected image and the solid line is from the corrected image. (g) and (h) are simulations of the PSF. Images based on [9].

The first is the effect of uncorrected aberrations from the sample and the optical path, which decrease the maximum intensity at the cover slip, but in a way that does not add linearly to the depth aberration. Thus only a fraction of the dispersed photons can be restored to the central peak. In closed loop AO systems, system aberrations are automatically compensated at each position (Wright et al., 2007), but in an open-loop system this is not possible. The second factor is the inability of the mirror to precisely

conform to the shape given by Eq. (1). The residual error of the mirror shape increases with depth (see Fig. 3c) so that as the imaging plane goes deeper and the possibility for improvement becomes greater, the improvement in peak intensities decreases

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.

[9]

[1] [6] [5]

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3.2 Point Scanning Microscopes

Scanning optical microscopes are widely used for high resolution imaging, mainly because certain implementations provide three-dimensional resolution with optical sectioning and are thus particularly useful for imaging the volume structures of biological specimensz. In these microscopes, illumination is provided by a laser that is focused by an objective lens into the specimen. The light emitted from the specimen is collected, usually through the same objective lens, and its intensity is measured by a single photodetector. The focal spot is scanned through the specimen in a raster pattern and the image is acquired in a point-by-point fashion. The resulting data are stored and rendered as images in a computer.

Several other point scanning microscope modalities have been introduced, including two-photon excitation uorescence (TPEF) microscopy, second harmonic generation (SHG) and third harmonic generation (THG) microscopy, and coherent anti-Stokes Raman (CARS) microscopy.

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3.2.1 Confocal Microscopes

The most common example of this type is the confocal microscope, which can be operated in reflection or fluorescence mode. Three-dimensional resolution is achieved by the placement of a pinhole in front of the photodetector. In a reflection mode confocal microscope, the illumination is scattered by objects not only in the focal region, but throughout the focusing cone. In fluorescence mode, emission is generated in the focus but also in out-of- focus regions. The pinhole ensures that mainly light from the focal region falls upon the detector and light from out-of-focus planes is obscured. It is critical in the confocal microscope that both the illumination and detection paths are diffraction limited. This ensures that i) the illuminating focal spot is as small as possible, and ii) that the focus is perfectly imaged on to the detector pinhole. Therefore, in an adaptive confocal microscope, aberration correction must be included in both paths. This dual pass adaptive system can usually be implemented using a single deformable mirror, if the path length aberrations are the same for both the illumination and the emission light. This is the case if there is no significant dispersion in the specimen or chromatic aberration in the optics.

A pinhole is not required to obtain three-dimensional resolution, so most TPEF microscopes use large area detectors to maximise signal collection. Although they rely upon other physical processes, non-linear imaging modalities such as SHG, THG and CARS exhibit similar resolution properties. When using large area detectors, the fidelity of imaging in the detection path is unimportant so the effects of any aberrations in this path are negated. It follows that single pass adaptive optics is appropriate for these microscopes as aberration correction need only be implemented in the illumination path.

Adaptive optics systems have been successfully combined with several point- scanning microscope systems including confocal,13 TPEF,6, 14, 15 harmonic generation,16, 17 CARS.18 Example images of aberration correction in an adaptive THG microscope are shown in Fig. 10.

[14] [4]

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3.2.2 Two-Photon Fluorescence Microscopy

[15] [7] [11]

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3.2.3 Harmonic Generation

[8] [13]

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3.2.4 CARS

[18]

4 Future Prospects

More work must be done before AO can become a regular component of laboratory microscopes. Most AO microscopes are too complex to set up, and their application can be limited by the robustness of operation. The development of automated alignment and calibration procedures would enable the turnkey operation needed to make these systems more practical.

The effectiveness of AO microscopy is mostly compromised by aberration measurement, rather than by currently available correction devices. More sophisticated wavefront sensors or sensorless optimization schemes will extend the microscope's ability to cope with large and more complex aberrations. An obvious goal is to develop "realtime" aberration sensing to increase the speed of correction. Coupled to this is the desire to reduce the exposure of specimens during the measurement process—an essential step when using microscopes for live imaging.

Aberrations can change significantly across a single field of view because the refractive index of the specimen varies throughout its volume. So far, the methods used in adaptive microscopes have provided only a fixed aberration correction for each image. This is sufficient if the imaged region is small enough that aberrations do not vary significantly across the field.

One way to overcome this limitation would be to apply multiconjugate AO to microscopes. This method has been applied in astronomy using multiple deformable mirrors to compensate for multiple aberrating layers in the atmosphere. A similar approach in microscopy would compensate for the 3-D refractive index distribution, although the optical system would become considerably more complex.

Further advances in AO will extend the capabilities of high-resolution microscopes to reveal functional and structural information from deep within biological tissue. Currently, optimum performance is often limited to thin regions near to the coverslip, sufficient for imaging individual cells, but of rather limited practicality for tissue imaging. AO promises to help move microscopy into a new regime in which biological studies that were previously confined to cell cultures can be performed in thick tissue and even in live specimens.

5 Conclusion

abberations that are important for imaging system differ a lot, some need correction of both illu and imaging, some use direct, other indirect methods, some only need correction of some abberation modes, others need as much as possible...important to choose correct system for given problem and figure it our in detail before ordering something

AO is applicable to almost all microscopy techniques...

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