Adaptive optics from microscopy to nanoscopy

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ABSTRACT

Adaptive optics have been introduced to compensate the effects of aberrations in high resolution microscopy. Adaptive elements, such as deformable mirrors or spatial light modulators enable the dynamic correction of aberrations in a range of applications. These methods have proved particularly useful in three dimensional imaging of tissue specimens. We review the principles behind these methods and their application to high and super resolution microscopy.

Keywords: Aberrations, adaptive optics, wave front sensing, confocal microscope, multiphoton microscope, STED.

1. INTRODUCTION

Optical microscopes are used for a wide range of applications, particularly in the biomedical sciences, where their ability to elucidate structure and function is unparalleled. Methods such as confocal and multi photon microscopy are particularly useful, as they provide three-dimensional resolution of volumetric structures. Coupled with the versatility of fluorescent marker technology, they are invaluable tools in the modern laboratory. The performance of these microscopes is however compromised by aberrations - distortions in the wavefronts of the light - which reduce the resolution and contrast of the images. Aberrations can be introduced by imperfect optics, such as misaligned lenses, or by the optical properties of a specimen. Differences in refractive index between the specimen mounting material, the coverglass, and the immersion medium can introduce spherical aberration, whose magnitude increases with depth. Further, more complex aberrations are induced through the variation of refractive index throughout a specimen. As the light propagates to and from the focus, parts of the wavefront travel through regions of differing refractive index and hence travel at different speed. Consequently, wavefronts are distorted and the focus becomes blurred.

The technique of adaptive optics (AO) was introduced to overcome these problems.[1–3] AO was originally conceived for astronomy, in order to overcome the aberrating effects of atmospheric turbulence on telescope imaging. Through measurement of the wavefront aberrations and their correction with an adaptive element, one could in principle remove the distortions and restore optimum imaging quality. In recent years, AO has been applied to other areas, such as ophthalmology, optical coherence tomography, laser based fabrication and microscopy. Advances in AO microscopy have resulted in a range of demonstrations of the benefits of this technology for improving high resolution imaging. This has required several innovations, complementing the AO methods employed in astronomy and other areas.

We present an outline of developments in the application of AO to microscopy. This includes an overview of technical considerations for the design of AO microscopes and description of practical implementations. The combination of AO with super resolution microscopy – or nanoscopy – is presented and in particular for stimulated emission depletion (STED) microscopy.

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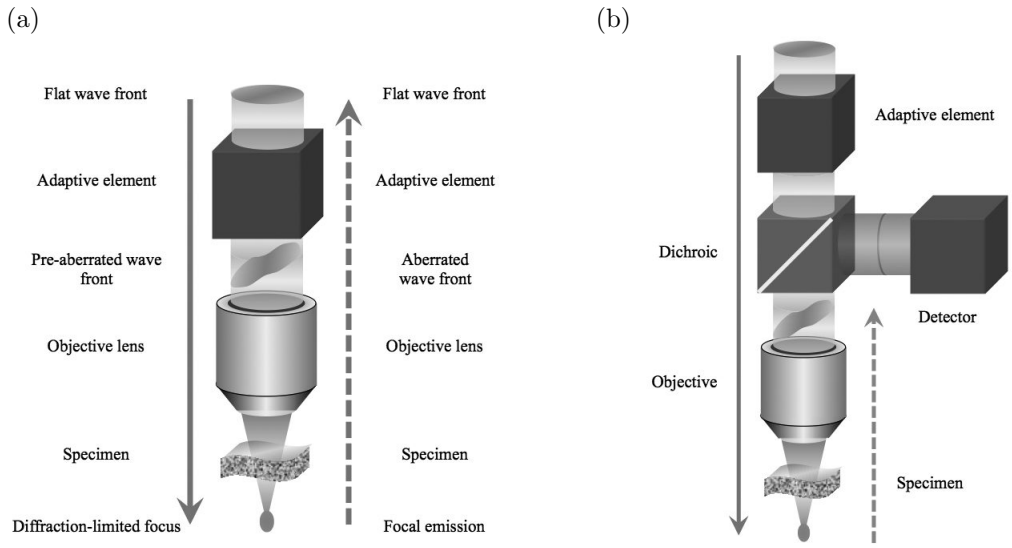

Figure 1. Schematic illustrations showing the placement of adaptive correction elements in different microscopes. (a) The placement of an adaptive element such as a deformable mirror in the path common to illumination and detection. This configuration would typically be employed in a confocal microscope. (b) A single pass configuration, as could be used in a multi-photon microscope using a spatial light modulator.

2. EFFECTS OF ABERRATIONS

Aberrations detrimentally affect the focussing properties of a microscope. In a laser scanning system, such as a confocal or multi photon microscope, the focal spot is distorted and reduced in intensity. The imaging of this spot onto the detector is also affected. In a multi photon microscope, where a large area detector is usually employed, these aberrations in the detection path have no effect on the final image quality. However, in the confocal microscope, where the emitted fluorescence must be imaged onto the pinhole detector, the fidelity of the detection path is important and aberrations do have a negative effect. Conventional widefield microscopes are only affected by aberrations in the imaging path. Indeed, it is well known that the aberrations of a condenser lens in the illumination path of a conventional microscope have no effect on the image quality. In other microscopes with more complex optical systems, aberrations can potentially affect all beam paths. These considerations have an effect on the design of the AO system and in particular on the placement of the dynamic correction elements. Example illustrations are shown in Fig. 1.

3. CORRECTION DEVICES

For microscopy, the main choice of correction element is between a deformable mirror (DM) or a spatial light modulator (SLM). There exist various deformable mirrors based upon different operation principles, such as electrostatic, electromagnetic or piezo-electric actuation. In most cases, the DM consists of a continuous membrane whose shape can be controlled by sending appropriate control signals to the actuators. Other DMs use segmented mirror elements rather than a continuous face sheet. As light is reflected off the DM surface, parts of the wavefronts must travel along a longer optical path than others. This results in an additional distortion of the wavefront caused by the DM shape. Through appropriate choice of the mirror control signals, one can add a distortion that is equal and opposite to the existing aberration in the incoming beam, thus correcting the aberration.

Spatial light modulators are usually in the form of pixellated liquid crystal devices, although micro mirror arrays are also available. For liquid crystal devices, a change in optical path length is effected by changing the configuration of the liquid crystal material through the application of a drive voltage. Reorientation of the liquid crystal molecules causes a change in effective refractive index, thus changing the speed of wavefront propagation

through the material. This has the effect of introducing an additional controllable distortion to a wavefront, which can be used to correct any aberrations.

Deformable mirrors have the advantage in microscopy that their operation is independent of wavelength and polarisation. This is useful in fluorescence microscopes, where the emission is broad band and randomly polarised. A single DM can also be used to correct both illumination and emission paths, even if they use different wavelengths, as the path length aberrations from refractive index variations in the specimen (assuming there is no significant dispersion) are readily compensated by the path length correction provided by the DM.

Spatial light modulators are normally designed to operate with polarised light, so are not optimised for use with fluorescence emission. Whilst SLMs usually have limited correction range - often around one wavelength or 2π radians of phase - they can be used to correct larger aberrations through the use of phase wrapping, whereby phase values ϕ of larger than 2π are "wrapped" back into the range $0 \leq \phi < 2\pi$. This approach is useful for narrow band light, such as laser illumination, but can lead to chromatic effects for broadband light, such as fluorescence emission.

4. ABERRATION MEASUREMENT

In AO microscopy, aberration measurement has been implemented using either wave front sensors or through indirect optimisation methods. Any wavefront measurement for 3D microscopy requires a method for excluding the effects of out-of-focus light, so that the detected aberrations are those induced on the path to (or from) the focal plane. For direct wavefront measurement, this can be achieved through methods such as pinhole filtering[4] or coherence gating.[5] Alternatively, one can ensure that the light used for sensing only originates in the focal plane through selective fluorescent marking of the specimen at isolated points[6,7] or using two-photon excitation, which is inherently confined to the focal point.[8] Indirect or "sensor less" wavefront measurement uses a sequence of intentionally aberrated images to provide an estimate of the initial aberration.[9] In this case, if the microscope provides three-dimensional resolution, then the light contributing to the the wavefront measurement process is already pre-selected as emanating from the focal plane. This method therefore ensures that the aberrations measured are only those that are induced through to the focus.

5. IMPLEMENTATION OF ADAPTIVE OPTICS IN MICROSCOPES

Adaptive aberration correction has been implemented in various microscopes with biomedical applications ranging from developmental biology to neuroscience (Fig. 2). The AO technology has also been used for microscopical applications in the analysis of three-dimensional photonics devices. These demonstrations have shown the ability of aberration correction to improve the resolution and contrast of images when focussing deep into specimens using different imaging modalities.

The preferred implementation for the confocal fluorescence microscope employs a single deformable mirror that corrects both the illumination and emission paths simultaneously.[10] This configuration ensures that the quality of the illumination focus and the fidelity of imaging from the focal emission to the pinhole detector is maintained. In two photon fluorescence microscopes, where a large area detector is used, only the aberrations introduced in the illumination path affect the image quality. For this reason, either a DM or an SLM can be used for compensation.[9,11] The SLM is suitable in this microscope, as it is only required to modulate the laser illumination, which is narrowband and linearly polarised. Similar configurations can be used for other multi photon methods, including harmonic generation microscopy. In wide field microscopes, AO correction need only be implemented in the imaging path. As these microscopes invariably operate with incoherent, unpolarised light (whether in transmission or fluorescence mode) a DM is usually the most appropriate correction device.[12]

6. IMPLEMENTATION IN SUPERRESOLUTION MICROSCOPY

Recently, AO has been developed further for application in super resolution microscopy. These methods use combinations of optical effects and/or photo-physical physical phenomena to enhance the effective resolution of a fluorescence microscope beyond the conventional diffraction limit. One prominent method is stimulated emission depletion (STED) microscopy, which can provide, in theory, unlimited resolution and in practice regularly leads

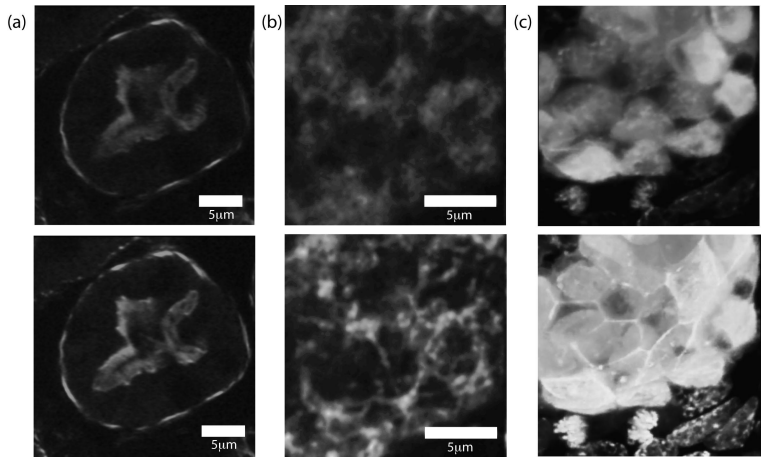

Figure 2. Examples of aberration correction in conventional resolution microscopy. Top row: before correction. Bottom row: after correction. (a) Confocal fluorescence microscopy of mouse kidney section. (b) Two-photon microscope images of GFP labelled *Drosophila* brain tissue. (c) Projections of 3D renderings from two-photon microscope images of a GFP and DAPI labelled mouse embryo.

to resolutions in the range of tens of nanometers in biological specimens.[13] In this microscope, fluorescence is excited in the specimen, as it would be in a confocal microscope, with a focussed laser beam. A second laser beam at a longer wavelength creates a ring-shaped focus that has a point of zero intensity at its centre, which is superimposed on the position of the excitation focus. This shaped focus is usually created by the introduction of an appropriately shaped phase mask into the pupil plane of the objective lens. The purpose of this second beam is to de-excite the fluorophores through stimulated emission and thus prevent them from emitting in the normal way. Emitted fluorescence is collected through the objective lens and passed through a pinhole to the photodetector, as it would be in the confocal microscope. As the stimulated emission process can be saturated, it is possible to increase the power in the depletion beam until only a narrow selection of excited fluorophores remains at the position of the intensity zero. In this way, the effective resolution of the microscope can be squeezed to a size much smaller than the original excitation focal spot. This principle has been applied to resolution enhancement in one, two and three dimensions, using appropriate phase masks for each case.

The resolution of the STED microscope depends primarily upon the quality of the ring-focus. A particularly important aspect is the value of the intensity minimum at the centre of the focus, which in practice is not perfectly zero. The way in which aberrations affect this intensity minimum and the surrounding bright ring is somewhat complex and depends upon whether the STED microscope is operated in the one, two or three-dimensional configuration. Some aberration modes have the effect of reducing the intensity of the ring, but maintaining a zero intensity at the centre (e.g. spherical aberration in 2D STED). Other modes can distort the distribution of the ring focus (e.g. coma), whereas certain modes lead to a filling in of the zero (e.g. astigmatism). Distortion and broadening of the depletion focus leads to a reduction in spatial resolution. A non-zero minimum intensity means that the STED effect also occurs at the centre of the depletion focus. In this case, it is possible that the majority of the excited fluorescence is depleted, meaning that no signal is recorded. This means it is possible that aberrations in the STED microscope lead not only to a reduction in resolution, but potentially also a loss of any useful image.

In the STED microscope, specimen induced aberrations affect three beam paths: the excitation path, the depletion beam and the emission path. As the imaging properties are determined in the first instance by the depletion beam, correction of aberrations in this path is most critical. For this reason, AO STED microscopes have been implemented using a single SLM placed in this beam path.[14,15] The SLM can be used for both aberration correction and as the phase mask required to shape the ring focus. This configuration was used to perform feedback correction of aberrations introduced by thick (around 10 to 20 μm) tissue specimens.

In order to compensate the effects of aberrations in all three beam paths, we have implemented a STED

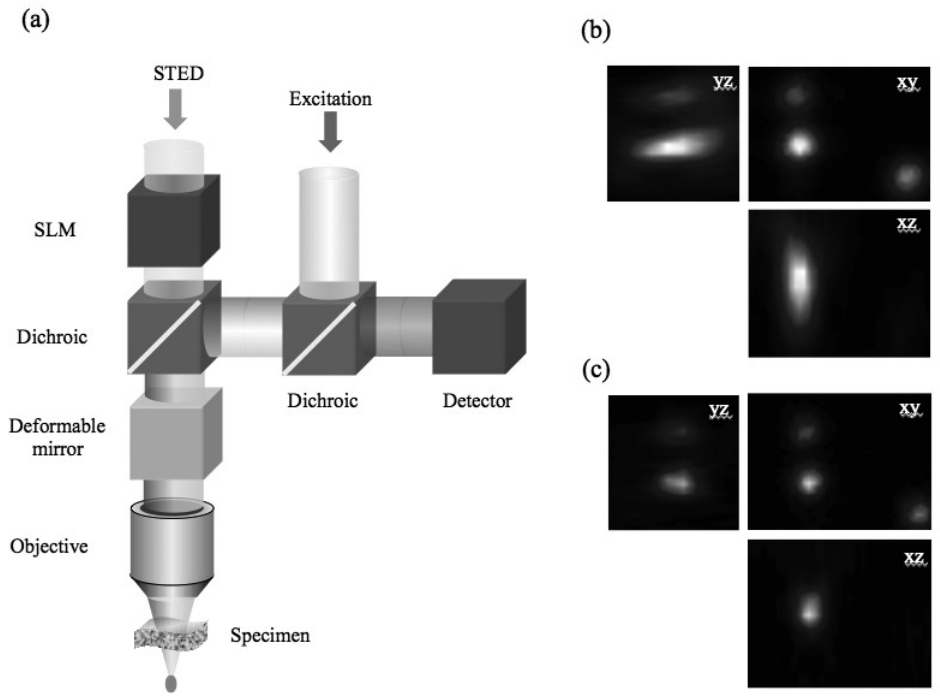

Figure 3. (a) Schematic diagram of the adaptive STED microscope employing both a deformable mirror and a spatial light modulator. (b) Orthogonal sections through 3D image stacks of 100nm beads imaged in confocal mode (depletion beam off). Aberrations have been partially corrected using the deformable mirror. The full width half maximum (FWHM) of the bead image in x is 252 nm, FWHM in z is 652nm. (c) 3D STED images of the same beads using additional aberration correction implemented using the SLM. The FWHM in x is 194 nm, FWHM in z is 247 nm.

microscope that incorporates both a DM and a SLM (Fig. 3). The SLM is again used in the depletion path; the DM is located so that it can correct all three paths simultaneously. The DM provides coarse aberration correction for all paths, whereas the SLM provides the phase mask and more precise aberration correction for the depletion beam alone. This configuration is better suited to correct of larger amplitude aberrations that may arise when focussing deeper into tissue specimens.

7. CONCLUSION

The recent progress in the development of AO techniques for microscopy has led to significant demonstrations of the use of aberration correction for imaging thick specimens. This technology will to help move biomedical microscopy into a new phase where studies that were previously confined to cell cultures or thin tissue sections can be performed in thick tissue. Furthermore, the adaptive methods will enable further advances in live specimen imaging. The adoption of AO as a widely used imaging tool will be assisted not only by further practical demonstrations, but also by development of more robust and efficient turn-key systems.

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