Characterisation of the effects of optical aberrations in single molecule techniques

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Abstract: Optical aberrations degrade image quality in fluorescence microscopy, including for single-molecule based techniques. These depend on post-processing to localize individual molecules in an image series. Using simulated data, we show the impact of optical aberrations on localization success, accuracy and precision. The peak intensity and the proportion of successful localizations strongly reduces when the aberration strength is greater than 1.0 rad RMS, while the precision of each of those localisations is halved. The number of false-positive localisations exceeded 10% of the number of true-positive localisations at an aberration strength of only ~0.6 rad RMS when using the ThunderSTORM package, but at greater than 1.0 rad RMS with the Radial Symmetry package. In the presence of coma, the localization error reaches 100 nm at ~0.6 rad RMS of aberration strength. The impact of noise and of astigmatism for axial resolution are also considered. Understanding the effect of aberrations is crucial when deciding whether the addition of adaptive optics to a single-molecule microscope could significantly increase the information obtainable from an image series.

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A wide range of fluorescence imaging techniques have been developed over the last few decades, with significant advances more recently in the spatial resolution achievable with the advent of super-resolution techniques such as single molecule localisation microscopy (SMLM) [1–4] and stimulated emission depletion (STED) microscopy [5]. However, these improvements have not been matched by an increase in the depth at which images can be obtained. The trade-off between depth and spatial resolution has led to super-resolution imaging being largely confined to studies of very thin samples or the superficial layers of thicker samples. The principal reason for this, particularly with biological samples such as cells, is that optical aberrations severely degrade image quality beyond the surface layer. These aberrations arise from mismatched and inhomogeneous refractive indices, within both the sample and the microscope as well as between the two, and increase with depth within the specimen [6]. Aberrations within the microscope result from imperfect manufacturing processes, imprecise alignment of optical components and optical design. In fluorescence
microscopy generally, the effect of aberrations is to reduce the signal-to-noise ratio and distort objects in the image plane, thereby reducing the spatial resolution.

Adaptive optics is widely used in astronomy to correct for aberrations caused by turbulence in the atmosphere [7]. Its application in microscopy [8] has been more limited and most work has been in scanning microscopies, including STED microscopy [9], where the appropriate aberration correction can be determined by a combination of image sharpness and brightness and can be modified pixel by pixel. In widefield microscopy, the challenge is greater because the aberration correction will generally apply to the whole field of view. Without prior knowledge of the structures present in a sample, it is difficult to know what the appropriate aberration correction is for all points in the field of view and all depths. Aberrations have been mapped in cells and thick tissue samples using scanning microscopy, but with relatively low resolution (10x10x10 µm³) [10, 11]. One technique that has been used in widefield imaging, analogous to the laser guide star in astronomy, is to insert a fluorescent bead into the sample [12]. However, it is challenging to position the bead in an appropriate location in cells and the fluorescence emitted from it may mask the organelles of interest. The fluorescence from endogenous or expressed fluorophores concentrated in small cell organelles have also been used to perform this function [13, 14]. In single molecule imaging and SMLM, we acquire multiple images of single molecules. The aberrations limit the number of single molecule features detected and their localisation precision. However, since we have prior knowledge of the unaberrated image of a single molecule, those molecules could themselves potentially be used as guide stars. A quality metric computed from raw images of multiple single molecules have recently been used to determine appropriate aberration corrections for SMLM of microtubules [15, 16]. Others have developed methods to correct for the aberrations from the microscope itself and refractive index mismatching that become noticeable with the resolution of SMLM [17–19].

The extent to which aberrations affect the analysis of raw data in single-molecule imaging techniques has not previously been systematically studied. The effects of selected aberration modes on the form of the point-spread function have been considered, but these studies were not extended to consideration of the effects on the final image [20, 21]. As we have discussed, measuring the aberrations present in a sample is a challenge in itself, but there is a need to understand the extent to which aberration correction is necessary. The effects of noise on aberrated single-molecule images should also be considered. Finally, one of the methods of obtaining axial resolution in single-molecule imaging is to add an aberration – astigmatism [22] – and so it is also important to investigate how unwanted aberrations affect 3D data obtained in this way.

2. Simulations

Optical aberrations can be characterised using Zernike polynomials. We simulated data sets containing aberrated point spread functions and considered different low-order Zernike modes in turn, at various strengths typical of cell and tissue samples [10, 11]. These data sets were then processed using a single molecule analysis package which revealed the significance of different aberrations on feature detection rates and apparent intensities in the poor signal-to-noise ratio environment of biological cells.

Similarly, we analysed the data sets using superresolution analysis packages to investigate the impact of aberrations on the achievable localisation precision and accuracy of the virtual image, and the likelihood of observing unwanted artefacts in the final reconstruction.

To create the simulated data sets, software was written using Interactive Data Language (IDL) that generates ten aberrated images, each containing a different random field of 100 spots representing single molecule fluorophores (Fig. 1) separated by at least 2.72 µm. In the case of a linear system invariant by translation, the image I of an object O is:

\[ I = \text{PSF} \ast O + n, \]

where \( \ast \) denotes the discrete convolution and \( n \) the noise of the recording.
process. The single molecule fluorophores (i.e. objects O) are modelled by Gaussian profiles and n by Poissonian photon shot noise followed by Gaussian detector read noise applied across the field of view. The point spread function (PSF) is equal to the absolute value-squared of the Fourier transform of the complex amplitude $\Psi$ of the electromagnetic field in pupil P:

$$\text{PSF}(r) \propto |\text{FT}[P(\xi)\Psi(\xi)]|^2,$$

where P is the binary pupil mask and $\xi$ is a distance vector in the pupil plane. The complex field in the pupil can be directly calculated by $\Psi = AP^{\phi}$, where A is the amplitude and $\phi$ the phase of the electromagnetic field in pupil P.

In this paper we only assume pure wavefront errors, without any amplitude variations in the pupil. The simulations used a fixed 515 nm fluorescence wavelength with each molecule emitting 400 detectable photons per image. Aberrated PSFs (generated using individual low-order Zernike polynomials) were then convolved with each of the resultant spots. These represent the aberrations experienced within the biological sample, assuming a spatially invariant PSF. A 100x 1.45 NA microscope objective, detector pixel size of 16 x 16 µm$^2$ and a total gain of 20 were used throughout the simulations, typical of commercial and custom-built instruments such as the Elyra microscope (Zeiss) equipped with an EMCCD detector (iXon, Andor). The gain, calculated using Andor’s data sheet and in digital counts per detected photon, is a typical value for iXon EMCCDs operating at 10 MHz readout rate, x250 EM gain, ~x4.7 pre-amp gain and 14 bit analogue-to-digital conversion. Lastly, Poissonian photon shot noise followed by Gaussian detector read noise (55 electrons from Andor’s data sheet) were applied across the field of view. Ten randomly generated fields of view, each with a single low-order aberration applied, were combined in stacks of increasing aberration strength. Tip and tilt were ignored because they correspond to a lateral shift in x and y respectively and do not contribute to feature distortion. Piston does not impact formation and was also ignored.

![Diagram](image)

Fig. 1. Formation and processing of simulated single-molecule fluorescence images. A random field of single molecules – the ‘ground truth’ – is convolved with aberrated point-spread functions with a range of aberration strengths, and noise added. Following localisation by a single molecule software package, the calculated coordinates are compared with those of the ground truth.

As noted in the introduction, an advantage of using adaptive optics is the possibility of applying a low level of artificial astigmatism to the emission path of the microscope. This allows localisation in the z-axis, enabling three dimensional imaging. We therefore also investigated the effects of each of the aberrations on 3D data sets in which localization discrimination along the optical axis z is obtained using astigmatism. 0.06λ RMS (i.e. ~0.38
rad RMS) of astigmatism was applied to each spot, as used in ref [23], and then different levels of defocus applied to simulate distances of 0, 250 and 500 nm into the sample.

In addition to the Poisson and readout noise present in a fluorescence experiment, we also studied the effects of increasing levels of background signal, and its associated noise, from within the specimen, for example due to auto-fluorescence. This was achieved by applying constant background offsets of 0-8 photons per pixel prior to the shot noise and read noise.

Aberrations in real samples do not, of course, always occur singly, but rather in combination. Simulations were therefore also produced using reported values of sample-induced aberrations from cells when imaging microtubules at a depth of 6 µm (Fig. 8(b) in ref [15]), with 100 frames of 100 randomly positioned aberrated point spread functions across a single imaging plane.

One of the most frequently used parameters to characterize the quality of optical image formation is the Strehl ratio. By definition, the Strehl ratio [24] is the quotient of the peak intensity of an aberrated point spread function (PSF) to the ideal diffraction-limited PSF, a value of 1.0 signifying perfect optical quality. A good approximation of the Strehl ratio for well-corrected optical systems [25] (i.e. as long as the true Strehl ratio is larger than approximately 0.3) can be obtained by $S = e^{-\frac{\sigma_\phi^2}{2}}$, where $\sigma_\phi^2$ is the variance of the phase aberration across the pupil in rad RMS. We determined the quality of the simulated images by measuring the Strehl ratio of each point spread function affected by different aberrations and at various aberration strengths.

Data sets were analysed using existing single molecule localisation software packages to return performance metrics such as localisation success rates and mean localisation error from each type of applied aberration. We used ThunderSTORM [26] and Radial Symmetry [27] because they had the best performance, in terms of localization accuracy and image resolution respectively, in a recent comparison of software packages [28]. We also used ThunderSTORM to investigate 3D SMLM, as it is capable of analysing astigmatic point spread functions.

For each simulated data set, the co-ordinates of the original point sources (ground truths) were compared to the coordinates output by each software analysis package when the same points were localized following the addition of aberrations and noise. This allows the measurement of the mean distance of the localisation from the ground truth (the accuracy) and the standard deviation of the measured positions from their mean (the precision). A localisation is deemed successful if only one is present within a certain radius, which we term the localization radius, of the unaberrated simulated ground truth.

False-positive localisations are those where a localisation has been made outside of this radius or where multiple localisations occur within this radius around a ground truth. These contribute to poor accuracy and artefacts in resulting images and single molecule tracks. To be comparable to the localization precision in single molecule tracking and SMLM experiments, the radius has been set to 250 nm (the full-width half-maxima (FWHM) of the unaberrated simulated ground truth) and 20 nm respectively. In practice, the localization accuracy in real experiments is much poorer than this even with 2D data [28].

3. Results and discussion
3.1 Effect of aberrations on single molecule images

The detection of a single fluorescent molecule and the accuracy and precision of its localisation depend on several factors. Among these, aberration plays a key role especially when imaging deep into the specimen. Indeed, the shape of the point spread function (PSF) is modified by aberrations as shown in Fig. 2. Aberration leads to a reduction in the fluorescence intensity and hence to a smaller signal-to-noise ratio (SNR).
As mentioned, the Strehl ratio is a measure of the effects of aberration. There will therefore be a correlation between the Strehl ratio and the SNR. The ratio reduces with increasing aberration. Figure 3(a) shows the Strehl ratios of the simulated data sets with increasing aberration strength. The PSF intensity sharply reduces as the aberration strength approaches $\phi = 1.25$ rad RMS, as the energy is dissipated to greater orders of the point spread function. The Strehl ratio, most notably for spherical aberrations, fluctuates at higher aberration strengths as the energy redistributes between diffraction orders of the PSF (Fig. 2(a)). Figure 3(b) clarifies that the zeroth order intensity is greater in this case for the higher aberration strength.

3.2 Localisation success

Figures 4(a) and 4(b) show the percentage of ground truths successfully localised by ThunderSTORM, as a function of aberration strength, in single molecule tracking and SMLM scenarios. The form of the various curves echoes those of the Strehl ratio (Fig. 3(a)). This reflects the fact that the data analysis package fits a Gaussian profile to each spot in the image and, as is clear from Fig. 3(b), this is a poor model when the side lobes have a relatively high intensity. Figures 4(c) and 4(d) show the equivalent plots when the Radial Symmetry algorithm is used, which detects circular features, also a poor model in the presence of aberrations such as astigmatism. Note that, because we have used a relatively low photon
count, the number of positive localisations is small when the localization radius is small. The effects of coma (Fig. 4(e)) are shown with a larger localization radius because the mean measured position deviates from the ground truth, as discussed in the following section.

Fig. 4. Localisation success in the presence of aberrations. Proportion of successfully localised ground truths, after analysis using (a, b) ThunderSTORM, (c, d) Radial Symmetry and defining a localization as successful if it is within (a, c) 250 nm or (b, d) 20 nm of the ground truth location. (e) Proportion of successfully localized ground truths, in the presence of coma, within 480 nm of the ground truth.

3.3 Localisation precision and accuracy

While the mean apparent location is approximately co-located with the position of the ground truth for most aberrations, there is a dramatic displacement when coma is present (Fig. 5(a), 5(b)). As Figs. 2(b) and 5(g) illustrate, this is caused by the zeroth order peak intensity of the point spread function shifting in x or y as coma is applied in the same axis. Note that the precision of the localisation may be barely affected as coma increases, as the intensity of the main spot is little changed, while the accuracy of the localisation, and hence of the resultant single molecule tracks or super-resolution image, is significantly worsened. This shift is as previously noted with 3D single-molecule data with the double-helical point spread function [20].

The localisation precision of individual molecules, and hence the resolution of an SMLM image, is limited by the number of photons contributing to each localised spot [29]. The number of photons emitted by a single organic fluorophore is typically limited to a few
hundred photons per image integration time when single molecule tracking, up to a few thousand photons in SMLM [30], and hence their efficient collection is critical. However, they are rapidly dissipated by aberrations, which degrade the quality of the PSF by redistributing photons from its centre to the periphery. This affects both the localisation accuracy and the localisation precision by increasing the background signal level and reducing the SNR. Figure 5(c)-5(g) shows how the experimentally determined precision deteriorates sharply as a function of aberration strength, as fewer photons contribute to the zeroth order of the PSF.

Fig. 5. Localisation accuracy and precision. (a, b) Accuracy (along x-axis) after analysis using (a) ThunderSTORM, and (b) Radial Symmetry for localisations within 480 nm of the ground truth; (c-f) Precision using (c, d) ThunderSTORM and (e, f) Radial Symmetry for localisations within (c, e) 250 nm and (d, f) 20 nm of the ground truth; (g) Profiles of the plots in Fig. 2(b).
Artefacts in single-molecule techniques will occur when spots are false-positively identified and so contribute to the final tracks or image. This happens when the analysis package identifies side lobes of an aberrated spot as one or more spots in their own right and is particularly likely to happen when the PSFs begin to overlap as the aberration strength increases. Our analysis scheme will also identify molecules subject to strong coma as false-positive localisations due to the displacement of the spot location in the image from the ground truth. Figure 6 shows the increase in false-positive detection as the aberration coefficient approaches 1.25 rad RMS, where the Strehl ratio tends to zero. At this point, it becomes inappropriate to model the PSF using a Gaussian profile, leading to inaccurate identifications.

Fig. 6. False-positive detection. Mean number of false-positive single-molecule detections per frame using (a, c) ThunderSTORM and (b, c) Radial Symmetry for a localization radius of (a, b) 250 nm and (c) 480 nm. Note that the number of ground truths is 1000.

3.4 Depth encoding using astigmatism

In three-dimensional microscopy, high numerical aperture (NA) objectives are used to maintain the highest resolution and signal intensity. Unfortunately, they only give well-corrected images just close to the coverslip surface. Indeed, as the focal plane is moved deeper into the specimen, the image degrades because of differences between the refractive index of the sample and that of the immersion media. Imaging features inside a 20 µm deep live sample with an oil immersion lens can cause the peak intensity of the PSF to drop three fold [31]. For this reason, we extended the simulations to three dimensions to investigate how aberrations affect the quality of the image at different depths. We introduced a constant level of 45° astigmatism (0.06λ RMS i.e. ~0.38 rad RMS) and added different amounts of other aberrations [23]. We again used ThunderSTORM, but with an elliptical 3D Gaussian model instead of the 2D Gaussian model used for 2D data. We looked at the number of successfully localised particles at three axial positions (at the focal plane and at 250 nm and 500 nm) and for all the different low order aberrations. Amongst the aberration modes, localisation was
most greatly affected by defocus when we go deep into the specimen and trefoil aberrations had the smallest effect; these are shown in Fig. 7(a).

Fig. 7. Astigmatic single molecule detection at different depths using ThunderSTORM, with a localization radius of 250 nm. (a) Number of successful localisations at various depths in the presence of defocus or trefoil aberrations; (b) Mean number of false-positive localisations exhibiting astigmatism aberrations, as a function of sample depth; (c) Accuracy (along x-axis) and (d) lateral precision for PSFs exhibiting defocus or trefoil aberrations as a function of depth; (e) Profiles of the plots in Fig. 2(c), along the major axis of the elliptical PSF.

Depth aberration leads also to higher false-positive detection. Figure 7(b) demonstrates how false-positive localisations increase dramatically for astigmatic aberrations above 1.25 rad RMS as sample depth increases. While the displacement from the ground truths is unchanging (Fig. 7(c)), the lateral precision reduces significantly with depth (Fig. 7(d)) but is little affected by the addition of further aberration terms.

Aberrations also significantly affect the width of the PSF along its major axis. Figure 7(e) shows the profile plot for astigmatic PSFs at different focal planes. As depth increases, the profile becomes broader and no longer forms a single intense peak, which results in the fitting algorithms localising the features less precisely.

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3.5 Noise

In quantitative fluorescence microscopy, we want to measure the signal coming from the fluorophores used to label the object of interest in our specimen. However, the intensity values in the digital images represent not only the signal of interest but also noise [32, 33]. This causes variance in the intensity values, imprecision in measurements of pixel intensity values, and therefore a level of uncertainty in the accuracy of the measurements. Noise arises from a variety of sources. Background noise is statistical noise from out-of-focus fluorophores and from sample autofluorescence. Photon noise is caused by the random statistical fluctuations in individual photon arrival times and its magnitude depends on the signal level. It can be modelled as a Poisson process and added to the signal from the image after the background noise. Finally, readout noise is generated by the detector electronics and is sampled from a Gaussian profile applied with a constant standard deviation across the image.

All the data in this paper includes typical levels of readout and photon noise. In Fig. 8, we have investigated the effect of additionally including background noise on the number of successful localisations, when applying different levels of astigmatism. The number of detected features decreases rapidly when additional photons of background noise are added. The reduction in the signal to noise ratio also reduces the aberration range the algorithms are able to fit. This effect was similar for all other types of aberration (not shown).

3.6 Aberrations in biological samples

Fig. 8. Combined effects of aberrations and image noise on single molecule detection. With a 250 nm localization radius, the (a) proportion of ground truths successfully localized and (b) precision, with increasing levels of background noise in the presence of astigmatism in the x-axis, was determined using ThunderSTORM.

Fig. 9. Effects of aggregated aberrations typical of biological samples. Simulated point spread functions containing (left) no aberrations and (right) specimen-induced aberrations when imaging microtubules in cells. Red crosses mark localization co-ordinates.
Our simulations have so far considered each Zernike mode separately. Thick specimens show multiple modes of aberration at varying strengths [14] which when combined may significantly affect the ability to localize the resulting PSFs.

Simulations using the specimen-induced aberrations found within microtubules imaged at a depth of 6µm inside cells [15], were analysed using ThunderSTORM’s 2D Gaussian algorithm. The aberration values were 0.00 rad RMS for defocus, 0.65 for astigmatism (0°), 0.45 for astigmatism (45°), −0.20 for coma (0°), 0.25 for coma (90°), 0.0 for trefoil (0°), −0.05 for trefoil (30°) and −0.75 for spherical aberration. We found that the localisation success rate had a mean average of 85.0% across the 10,000 ground truths. The mean false-positive rate was more than 300% higher due to multiple fits to single PSFs (Fig. 9, right). This indicates that a resultant reconstructed SMLM image with no aberration correction could contain a large number of artefacts and could lead to anomalies in single molecule tracking. However, ThunderSTORM’s 3D elliptical Gaussian algorithm had a mean average localisation success rate of 80.6% with a 40.8% false-positive rate, indicating that a greater proportion of PSFs were only localised once.

4. Conclusion

The achievable resolution in fluorescence microscopy is limited by the presence of aberrations, especially in thick samples. Using simulations, we have shown that aberrations have a significant impact on single-molecule localisation and, consequently, on the resolution that can be achieved in SMLM and single molecule imaging. For clarity, we chose to consider the effects of different aberration modes individually, although in practice multiple modes will usually be present and hence we have also shown the aggregated effects of measured aberrations from a real sample. As aberrations increase in strength with depth, localisation success, accuracy and precision all reduce. However, adaptive optics can recover the optimal imaging properties of the microscope, maintaining them even when focusing at different depths into thick specimens. The achievable precision is also limited by the presence of different sources of noise. When noise is coupled with aberrations, the resulting image is even less precise. Increasing noise levels diminish the number of successful localisations at a given aberration strength, and additionally reduces the tolerance of the fitting algorithms to stronger aberrations. Our results will aid microscopists in deciding the extent to which adaptive optics will benefit them when using single molecule techniques.

As expected, we have shown that the number of positive localisations is strongly dependent on the type of aberration and the localization software used. Of the two software packages that we tried, Radial Symmetry showed the strongest resilience to aberrations, particularly in minimizing the number of false-positive detections. The precision is equally affected by the type of aberration and its strength. For the imaging configuration used in this paper and to maintain the precision level below 40 nm, aberrations must be below ~0.6 rad RMS – which is challenging for any adaptive optics system. However, a precision of 60 nm can still be achieved with aberrations of 1.0 rad RMS. The peak intensity and the proportion of successful localizations strongly reduces when the aberration strength is greater than 1.0 rad RMS, while the precision of each of those localisations is halved. The number of false-positive localisations exceeded 10% of the number of true-positive localisations at an aberration strength of only ~0.6 rad RMS when using the ThunderSTORM package, but at greater than 1.0 rad RMS with the Radial Symmetry package. In the presence of coma, the localization accuracy is unsurprisingly poor, reaching an error of 100 nm at ~0.6 rad RMS aberration strength. When an astigmatic PSF is used to achieve axial resolution, defocus has a large impact on localization, but other modes have a much smaller effect and the proportion of successful localisations is only significantly altered for trefoil when the aberration strength exceeds 2 rad RMS at a depth of 500 nm. A similarly predictable effect on the number of false-positive localisations is also seen, with a sharp increase at 0.6 rad RMS astigmatism aberration strength at a depth of 250 nm. Taken altogether, these simulations show that the
desirable level of aberration correction by adaptive optics is high. Keeping the aberrations below 0.2 rad RMS will enable high localization accuracy and precision at depths of 500 nm or more when noise levels are no more than a few photons per pixel.

We note that the software packages we used assume that the image of a single molecule is of fixed size and Gaussian in profile (and elliptical when astigmatism is added for axial resolution). Improved fitting algorithms could account for a range of aberrated PSFs, increasing the single molecule detection rate. Indeed, our tests with ThunderSTORM with real values for the Zernike mode strengths showed that an elliptical model for the PSF is more robust in the presence of aberrations than the more usual circular one. Using real aberration values also highlighted the number of false-positive localisations that can arise. In SMLM, these form groups with identical temporal characteristics, which could enable them to be averaged as a single molecule provided that the groups do not spatially overlap. In single-molecule tracking, where the aberrations are likely to change through time as a molecule moves around a biological cell and tracks cross, this is less practicable.

The model we have used in this work incorporates several simplifications, which an extension to this work could account for. Firstly, we chose to ignore scattering effects, common in biological samples. Secondly, a polychromatic model would account for the emission spectrum of a typical fluorophore, rather than the single wavelength used here. We used a scalar model and did not account for the vectorial nature of the electromagnetic field, which is particularly relevant to high numerical-aperture objective lenses. We generated aberrations in a single pupil plane, whereas sample-induced aberrations are actually generated throughout the sample volume. Although we used a simplified image formation model, it is still able to reproduce most of the features of an actual PSF.

We have described the advantages of using adaptive optics to correct for aberrations. Such corrections are usually applied to the entire field of view with the implicit assumption that the aberrations are spatially invariant. Whilst this may be the case in astronomy where sufficiently narrow fields of view are investigated, it is not the case in microscopy where sample-induced aberrations vary significantly across the specimen [11]. This means that deciding the area over which a homogeneous correction can be applied is a critical step [10] and depends on understanding the impact of aberrations on single-molecule data analysis. Moreover, in most implementations, the correction for spatially invariant aberrations is performed by placing the adaptive optics in the pupil plane. However, the part of the image within which the wavefronts are corrected can be much larger if the deformable mirror is placed in a conjugate plane [34]. Considering both pupil and conjugate plane positions for the adaptive optics could optimize aberration correction and hence image quality.

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