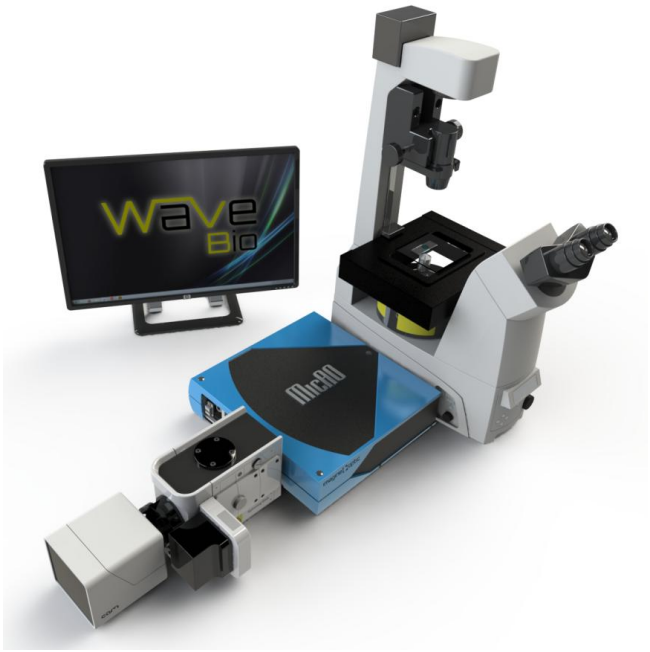


# Simple method to improve image contrast in spinning disk microscopy using adaptive optics

APPLICATION NOTE

**Grégory CLOUVEL, Audrius JASAITIS and Xavier LEVECQ**
**Imagine Optic, 18 rue Charles de Gaulle, 91400 Orsay, France**
**contact@imagine-optic.com**

## Summary

Aberrations induced by the optical components of the microscope, the refractive index mismatch and also aberrations induced by the biological sample, distort the point spread function (PSF) of the optical setup and reduce the amount of detected photons. This leads to impaired image quality, preventing accurate analysis of molecular processes in biological samples and imaging deep in the sample. The mismatch of refractive index has the biggest impact in terms of aberrations in spinning disk microscopy, where oil immersion objective is used to image deep layers of live biological samples, mostly composed of water. In this application note we demonstrate how adaptive optics device MicAO SD can be used to correct aberrations and improve the imaging quality. The module overcomes undesired anomalies by correcting for most of aberrations in confocal imaging. In this application note we present an aberration model, which allows automatic correction of spherical aberration when performing Z stacks of live biological specimen, resulting in significant increase of image contrast. We also demonstrate that correction of aberrations in the images also boosts the particle detection success rate by 25%.

Spinning disk (SD) microscopy is one of the most common imaging techniques with optical sectioning capability used in research laboratories for *live* imaging. This technology represents a state-of-the-art approach to study morphogenesis. It can resolve live biological processes on a multitude of spatial scales from sub-cellular to relatively thick tissues. SD induces low photo damage and allows for fast multicolor 3D imaging using a low-noise and high-dynamic-range detector such as EMCCD or sCMOS camera. Nevertheless, this type of microscopy is affected by aberrations, induced by the optical elements and, in particular, the mismatch of refractive indexes of objective lens's immersion medium and biological sample (Booth, 2007; Booth, 2014). To restore the optimum performance of SD method, Imagine Optic developed an adaptive optics device MicAO SD. This plug&play module, which contains a continuous membrane deformable mirror (Mirao 52-e) is inserted between the spinning disk unit and imaging camera. The dedicated software includes all the necessary routines to detect and correct aberrations, which we will discuss in this application note.

Two main strategies of aberration correction, currently used in microscopy, are the closed-loop and image-based iterative algorithms. In the closed-loop mode the wavefront sensor, is measuring the wavefront from a point source (a guide star) inside the inhomogeneous medium and distortions of the wavefront are then corrected by a phase modulator, such as deformable mirror. This method was used to correct aberrations in both fixed and live cell imaging in confocal and two-photon microscopy (Tao et al., 2011; Tao et al., 2012; Aviles-Espinosa et al., 2011; Wang et al. 2014). Unfortunately, a guide star typically is not present in biological samples or the intensity of light emitted by such guide star is not enough to measure the wavefront by ordinary CCD-based Shack-Hartmann wavefront sensor.

For this reason a number of image-based sensorless methods, such as pupil segmentation, genetic, 3N *etc*, have been developed to detect aberrations in different types of microscopy (Ji et al., 2010; Débarre et al., 2009; Kner et al., 2010; Facomprez, 2012). These methods do not require a wavefront sensor and for detection of aberrations are using images taken by imaging camera. They detect aberrations very effectively in fixed or slowly-changing biological samples and are currently used in scanning confocal and multiphoton microscopy. However, these methods require a lot of images to be taken to obtain a good correction and because of that they become not efficient for *live* imaging, mainly because of rapid cellular movements and fluorescence signal fluctuations from one image to another.

But in some specific conditions it is possible to predict aberrations and their amplitudes. Spinning disk confocal microscopy is a good example of such configuration. The typical samples in spinning disk microscopy are multi-cellular *live* biological samples, such as tissue slices or even small embryos, which are imaged by acquiring Z-stacks at different depths. The Yokogawa CXU-1 spinning disk device is optimized for a high magnification and high numerical aperture oil immersion objectives. Therefore there is a significant refractive index mismatch between the water-based biological sample (n = 1.36-1.40) and the immersion oil of the objective (n = 1.52). This mismatch induces spherical aberration, which is a dominant aberration in spinning disk imaging, distorting and blurring images at depth. The amplitude of spherical aberration increases with increasing depth (Diaspro et al, 2002) and this depth dependence can be predicted. The depth dependence of various aberrations has been modeled by Wilson's group in Cambridge (Booth et al., 1998) and recently this

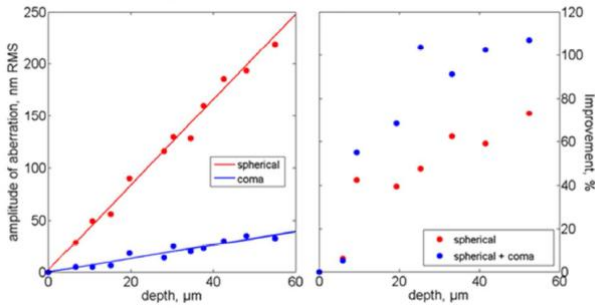


**Figure 1.** Experimentally-determined depth dependence of aberrations using a sample of fluorescent beads in agar. **Left panel:** Experimentally-determined depth dependence of spherical aberration (red) and coma (blue) in oil-water refractive index mismatch conditions. Solid lines are linear fits of the data. **Right panel:** Corresponding improvement of the fluorescence intensity when only spherical aberration (red) and both spherical aberration and coma (blue) are corrected.

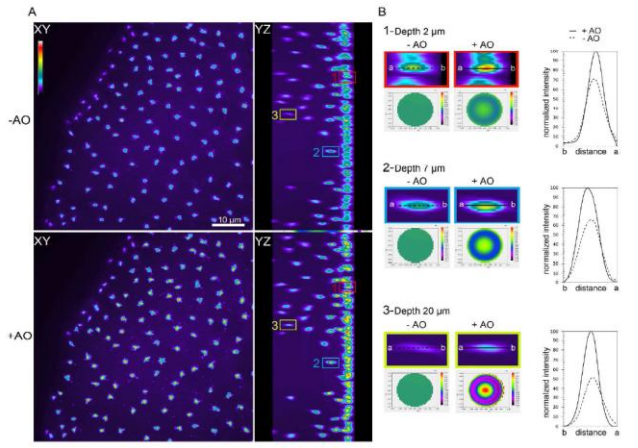


**Figure 2.** Comparison of gamma-tubulin-GFP on fixed *Drosophila* imaginal disk (centrosomes of epithelial cells) acquired with spinning disk microscope with (+AO) or without MicAO SD (-AO). **A.** Images on the left are the maximum intensity projections of 25 µm Z stack in XY plane. Images on the right show the XZ view of the stack. **B.** Comparison of wavefront measurements with and without AO at different depths. At each depth, the profile line with normalized intensities reveals improvement achieved with AO. The scale bar is for both XY and YZ.

model has been applied in STED microscopy (Lenz et al., 2014). We decided to address this question empirically.

To determine the depth dependence of Zernike modes in oil-water refractive index mismatched conditions, we constructed a model sample composed of diffraction limited fluorescent beads embedded at different depths in a thick 2% agarose gel with a refractive index close to water. We used the 3N image-based iterative algorithm (Débarre et al, 2009)[2] to determine aberrations by varying the major Zernike modes and using maximum intensity as the merit function. As expected in this refractive-index-mismatched configuration, the main detected aberration was the spherical aberration that showed a linear dependence in the examined depth range up to 60 µm (Figure 1, left panel). Unexpectedly, we also found a less pronounced linear depth dependence of coma, the correction of which also consistently improved the fluorescence intensity (Figure 1, right panel). For example, at a depth of 20 µm, the fluorescence signal improved by 40-50% after correction for only spherical aberration and by 70-80% after correction of spherical aberration and coma. This contribution of coma was found to be due to a small misalignment of the optical setup. The amplitudes of other Zernike modes were small (<10nm RMS), therefore their impact on the wavefront was neglected.

We validated our experimental model by imaging fixed *Drosophila* brain multilayered structure labeled for centrosomes (Figure 2). In such a sample the refractive index mismatch is particularly complex because each layer has different optical properties (Booth and Wilson, 2001) and our model ignores the distortions from the sample itself. Nevertheless, optimization of spherical aberration and coma clearly improved the image quality across the whole brain, with greater brightness and contrast, resulting in more distinct structures in corrected images. Even at 2 µm from the surface of the coverslip, where the 100x objective should be very efficient, the gain of 30% in intensity was obtained. At 20 µm depth, the effect of aberration

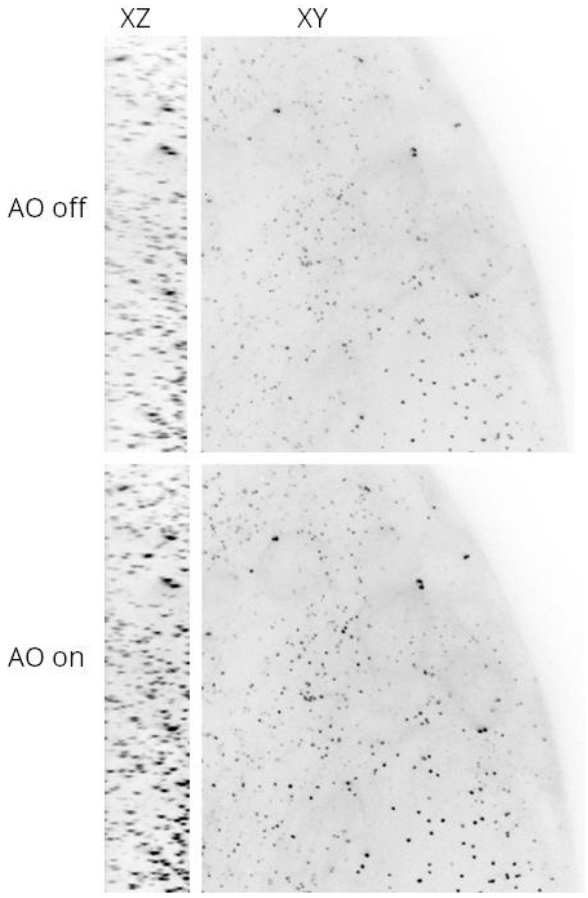


**Figure 3.** Comparison of Ubq-DSas-4-GFP (centrosome-reference) on 3D *Drosophila* brain imaged with spinning disk microscope with (AO on) or without MicAO SD (AO off). Images are maximum intensity projections of 31 planes in a Z stack.

correction was even better, resulting in a 2-fold improvement. Moreover, at depth the shape of the centrosome is also corrected and better preserved, allowing for a much better assessment and interpretation of the observed sample.

Similar results we also obtained by imaging live *Drosophila* brain samples (Figure 3) – the contrast of the images is significantly improved. In this case we decided to verify if the correction of aberrations can improve the statistics of particle detection. For the detection of centrosomes we used Spot Detector tool from Icy imaging software package (Institut Pasteur, Paris). Using the standard detection parameters, suggested by the developers of the software, we were able to detect up to 25% more centrosome particles in images corrected for aberrations.

Here we showed a new method of aberration correction in refractive index mismatched conditions, which is based on experimental characterization of various aberrations with depth and does not require any images for the detection of aberrations. This method is easy to operate and at the same time provides a robust and sample-independent improvement of the fluorescence signal, especially when deeper layers are imaged. This fast aberration correction method doubles the intensity of the fluorescence signal when biological samples are imaged either fixed or live.

## Acknowledgements

This work was supported by EU Seventh Framework Programme (FP7-ICT-2009.3.7: Photonics; Nexpresso call 1). We would like to thank Vincent Fraisier and Jean Salamero (Insitut Curie, Paris) for providing the optical setup, Julie Plastino (Insitut Curie, Paris) for reading and commenting on the manuscript, Dr. Maria-Alexandra Rujano Maldonado (Insitut Curie, Paris) for providing us *Drosophila* samples. We also thank Dr. Dora Sabino (Insitut Curie, Paris) for technical assistance with sample preparations.

## References

Aviles-Espinosa, R., Andilla, J., Porcar-Guezenec, R., Olarte, O. E., Nieto, M., Levecq, X., Loza-Alvarez, P. (2011) "Measurement and correction of in vivo sample aberrations employing a nonlinear guide-star in two-photon excited fluorescence microscopy." *Biomed. Opt. Express*, **2**, 3135. http://dx.doi.org/10.1364/BOE.2.003135

Booth, M. J., Neil, M. A. A., Wilson, T. (1998) "Aberration correction for confocal imaging in refractive-index-mismatched media." *J. Microsc.-Oxford*, **192**, 90. http://dx.doi.org/10.1111/j.1365-2818.1998.99999.x

Booth, M. J., Wilson, T. (2001) "Refractive-index-mismatch induced aberrations in single-photon and two-photon microscopy and the use of aberration correction." *J. Biomed. Opt.*, **6**, 266‑272. http://dx.doi.org/10.1117/1.1382808

Booth, M. J. (2007) "Adaptive optics in microscopy." *Phil. Trans. Royal Soc.,* **365**, 2829. http://dx.doi.org/10.1098/rsta.2007.0013

Booth, M. J. (2014) "Adaptive optical microscopy: the ongoing quest for a perfect image." *Light Sci. & Appl.*, *3*, e165. http://dx.doi.org/10.1038/lsa.2014.46

Debarre, D., Botcherby, E. J., Watanabe, T., Srinivas, S., Booth, M. J., Wilson, T. (2009) "Image-based adaptive optics for two-photon microscopy." *Opt. Lett.*, **34**, 2495. http://dx.doi.org/10.1364/OL.34.002495.

Diaspro, A., Federici, F., Robello, M. (2002) "Influence of refractive-index mismatch in high-resolution three-dimensional confocal microscopy." *Appl. Opt.*, **41**, 685. http://dx.doi.org/10.1364/AO.41.000685

Facomprez, A., Beaurepaire, E., Debarre, D. (2012) "Accuracy of correction in modal sensorless adaptive optics." *Opt. Express*, **20**, 2598. http://dx.doi.org/10.1364/OE.20.002598

Ji, N., Milkie, D. E., Betzig, E. (2010) "Adaptive optics via pupil segmentation for high-resolution imaging in biological tissues." *Nat. Meth.*, **7**, 141‑U84. http://dx.doi.org/doi:10.1038/NMETH.1411

Kner, P., Winoto, L., Agard, D. A., Sedat, J. W. (2010) "Closed loop adaptive optics for microscopy without a wavefront sensor." *Proc. SPIE*, **7570**, 757006. http://dx.doi.org/10.1117/12.840943

Lenz, M. O., Sinclair, H. G., Savell, A., Clegg, J. H., Brown, A. C. N., Davis, D. M., French, P. M. W. (2014) "3-D stimulated emission depletion microscopy with programmable aberration correction." *J. Biophoton.*, **7**, 29. http://dx.doi.org/10.1002/jbio.201300041

Tao, X., Azucena, O., Fu, M., Zuo, Y., Chen, D. C., Kubby, J. (2011) "Adaptive optics microscopy with direct wavefront sensing using fluorescent protein guide stars." *Opt. Lett.*, **36**, 3389. http://dx.doi.org/10.1364/OL.36.003389.

Tao, X., Crest, J., Kotadia, S., Azucena, O., Chen, D. C., Sullivan, W., Kubby, J. (2012) "Live imaging using adaptive optics with fluorescent protein guide-stars." *Opt. Express*, **20**, 15969. http://dx.doi.org/10.1364/OE.20.015969

Wang, K., Milkie, D. E., Saxena, A., Engerer, P., Misgeld, T., Bronner, M. E., Betzig, E. (2014) "Rapid adaptive optical recovery of optimal resolution over large volumes." *Nat. Meth.*, **11**, 625. http://dx.doi.org/10.1038/NMETH.2925