Airyscanning

Evoking The Full Potential of Confocal Microscopy

Confocal laser scanning microscopes (CLSMs) are renowned for their sectioning capability. This feature is enabled by the use of a pinhole, which rejects out-of-focus light. Less appreciated, on the other hand, is the gain in lateral resolution by this type of microscopes for one obvious reason. As the pinhole is closed to improve the resolution, less light can reach the detector leading to images with poor signal-to-noise ratios (SNR). Therefore CLSMs are operated with a pinhole diameter of around 1 Airy unit (AU), sacrificing resolution for SNR. But with a clever detector design one can solve this conundrum. By using a sub-Airy detector element array instead of a single point detector, it is possible to collect light more efficiently boosting the strengths of a confocal. The extra photon budget can be diverted towards increasing the sensitivity, the scanning speed or the resolution of the microscope. In this article we will focus our discussion on how resolution is improved.

Ralf Engelmann

Klaus Weisshart

Improving Resolution

The resolution in a widefield microscope is limited by the diffraction of light, which causes a point emitter to be imaged as an extended spot [1,2] (Fig. 1).

An improvement in resolution in fluorescence microscopy by a factor of 1.4 was achieved by confocal laser scanning microscopy (CLSM) [3]. The sample is hereby illuminated with a scanned focused laser beam. The detected intensity values of every scanning position are recorded with an integrating detector like a photomultiplier tube (PMT). The resolution enhancement is achieved by collecting the emitted light through a pinhole, which is aligned to the position of the excitation focus. That way all detected photons will be assigned to the nominal excitation position.

Despite this slight resolution improvement CLSM is more renowned for its optical sectioning capabilities for one very clear reason. The increase in resolution is only achieved by minimizing the pinhole diameter, theoretically down to zero. As a consequence detection efficiencies would be dramatically reduced and this would yield images with poor signal-to-noise ratios (SNR). Therefore, in practical terms, the pinhole radius will hardly be closed below one Airy unit, sacrificing resolution for SNR.

Resolution and Signal Improvement

The insight that a displaced pinhole detector produces an image of about equally improved resolution as an aligned pinhole detector, however smaller in amplitude and shifted by half the displacement (fig. 2), is the key for constructing a compound eye detector for a confocal microscope [4]. It allows arranging various pinhole detectors in an array covering an area exceeding the conventional pinhole size by approximately one quarter. Reassigning the detected photons from the shifted detector to the central detection position and summing up the back shifted signal from all detectors will increase the detection efficiency as no light is rejected by a closed pinhole but rather collected by the off-axis detector elements. Therefore an increased signal level arises from the reassignment of photons to a smaller spatial region [5].

One way to realize off-set pinhole detection would be to employ an array detector like a pixilated camera in place of

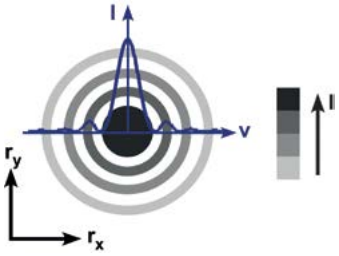

Fig. 1: Due to the diffraction nature of light, a point source will be imaged by a microscopic system as a blurred spot surrounded by rings of decreasing intensities (I) in the lateral plane (r_x, r_y), the so-called Airy disk (displayed in grey). The intensity distribution along a transverse direction (v) is shown in blue.

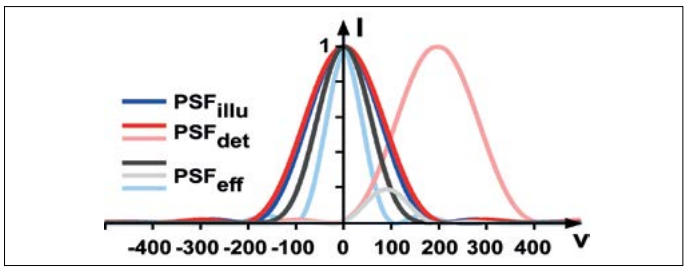

Fig. 2: The effective PSF (PSF_{eff}; dark grey) in a confocal microscope with aligned illumination and detection is the product of the illumination PSF (PSF_{illu}; dark blue) with the detection PSF (PSF_{det}; dark red). It will therefore be narrower. As the detector element is displaced in the transverse direction (v), the amplitude of the detection PSF (light red) will be shifted the same way. The resulting effective PSF (light grey) will shift by a smaller increment and has a decreased amplitude. But it will be narrower than is the case for the on-axis detector (compare dark grey with light blue PSF, the latter being the effective PSF derived by the displaced detector, but shifted back and normalized for better visualization).

Fig. 3: The object (grey spot) is illuminated by a point source (blue) and will emit photons that will be recorded on an array detector. As the sample is scanned (in the direction of the arrow), not only will each detector element (squares) see the whole image, but they would probe the Airy disk (displayed in orange) as well. The obtained detection PSFs (grey curves) would be shifted and their amplitudes decrease with increasing shift.

the pinhole. Indeed such a setup has been applied in single and multi-spot excitation schemes for imaging biological samples [6,7]. Another possibility would be an all-optical way where the detection beam after having been de-scanned is displaced from the optical axis by a defined amount via a re-scan [8, 9]. In all these methods based on pixel reassignment a lateral resolution enhancement by a factor of 1.7 has been obtained compared to a widefield image. Thus, most of the effect is the result of a greatly improved signal allowing the small pinhole sizes for the individual element of the detector; yet another tiny part stems from a slight improvement upon the true confocal resolution limit by narrowing the effective PSF. In pure reassignment mode axial resolution is lost [5]. However, it is possible to even improve axial resolution with proper algorithms.

Isotropic Resolution Increase in all Three Spatial Directions

In Airyscan a PMT point detector array is used with the benefit of a fast read out time compared to a camera and low dark counts. As the object is scanned all detector elements will record the whole image for each scanner position. Into the bargain, since at each scan position an image is taken by the detector array the whole Airy disk is acquired (fig. 3).

Pixel reassignment would now be one way to boost the lateral resolution. But since each detector element acts like an individual pinhole, each image can be individually deconvolved. A proper weight can be applied to the individual images and photons properly assigned. As axial information is accessible and not lost, an isotropic 1.7 fold increase in resolution in all spatial directions can be achieved.

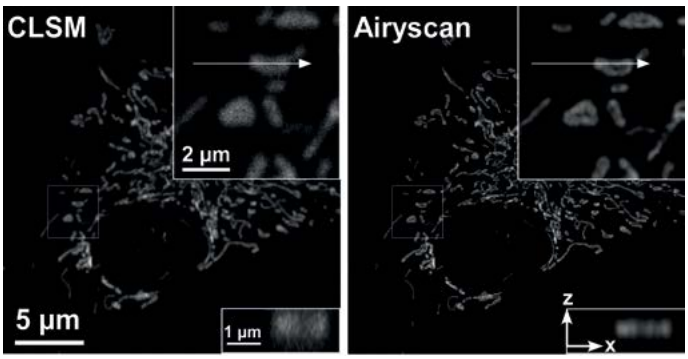

Fig. 4: Confocal (CLSM) and Airyscan recording of a FluoCell #1 (Invitrogen) imaged for MitoTracker to visualize mitochondria. Shown in the upper right corner is a magnified view of the area indicated by the smaller squared box. The lower right corner shows an xz section along the indicated arrow in the inset. Image conditions were identical with a pixel size of 58 nm and a sectioning of 125 nm. Scales are indicated.

A commercial realization of the concept is provided by Airyscan from Zeiss. Here, the detector design consists of a 32 channel gallium arsenide phosphide (GaAsP) PMT array arranged in a compound eye fashion. Each detector element will represent one specific offset position of the Airy function (see cover image of this issue). The integrated processing algorithms enable online deconvolution for visualization of resolution enhanced images, given proper 2.3 fold oversampling has been performed to meet the Nyquist criterion for the increased resolution (fig. 4). With conventional resolution, a virtual pinhole effect and sensitivity improvement can be obtained as alternative imaging modes.

Conclusion

The concept evokes the full potential of a confocal microscope obtaining resolutions comparable to a very small pinhole, but with a much better SNR. The method works for any dye and is live cell compatible, as low laser powers can be applied. Last not least as it is based on the confocal principle, thick samples are amenable to analysis that would fail with other widefield based super-resolution techniques. As such the method can offer an useful and complementary tool to study cellular structures to finer details.

References

[1] E. Abbe: Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung, Archiv Mikroskop Anat. 1873 (9) 413–468
[2] S. Hell and A. Schönle: Oxford University Press, 237–264
[3] J. Pawley: Handbook of biological confocal microscopy, Springer (2010)
[4] C. J. Sheppard: Optik 80 (2), 53–54 (1988)
[5] C. J. Sheppard *et al.*: Opt Lett. 38(15), 2889–2892 (2013)
[6] C. B. Muller and J. Enderlein: Phys Rev Lett 104(19), 198101 (2010)
[7] A. G. York *et al.*: Nat Methods 9(7), 749–754 (2012)
[8] S. Roth *et al.*: Optical Nanoscopy 2(5), 1–6 (2013)
[9] A. G. York *et al.*: Nat Methods 10(11), 1122–1126 (2013)

Contact
Ralf Engelmann
Tiemo Anhut
Ingo Kleppe
Klaus Weisshart

Carl Zeiss Microscopy GmbH
Jena, Germany
klaus.weisshart@zeiss.com