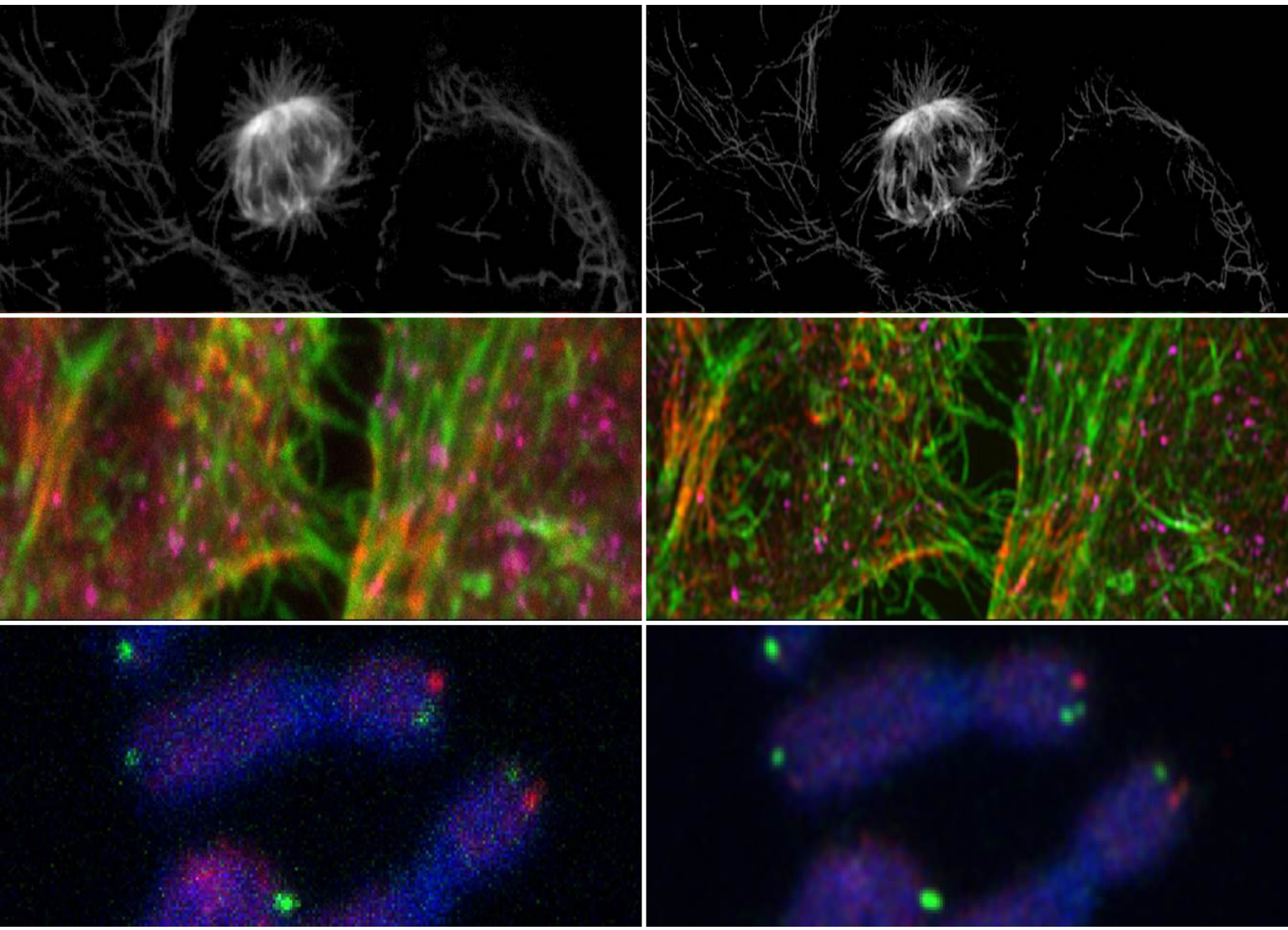
Technology Note



Airyscanning

A Novel Approach to Confocal Imaging

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Airyscanning

A Novel Approach to Confocal Imaging

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Confocal laser scanning microscopy (CLSM) is the recognized standard for 3D fluorescence microscopy. It combines excellent optical sectioning performance with flexible scanning strategies for imaging and photomanipulation, making it the method of choice for a vast range of applications. Airyscanning is a new detection concept that uses an array detector to oversample each Airy disk in order to gain sensitivity, resolution and speed.

Pinhole based laser scanning microscopy

In CLSM the sample is illuminated by a scanned focused laser beam. The emitted fluorescence signal is sent to a detector through a pinhole aperture which efficiently blocks out-of-focus light. Making the pinhole smaller increases resolution, yet at the same time less light reaches the detector, and - as a consequence - the signal-to-noise ratio (SNR) drops. In a typical experiment the pinhole will not be smaller than one Airy Unit (AU), sacrificing resolution for better SNR.

Limitations of classical CLSMs

Phototoxicity

The key challenge when imaging live samples is to maintain physiological conditions—especially for 3D acquisitions or long term time-lapse recordings.

While environmental conditions such as temperature or pH can be controlled by incubation systems, continuous exposure to excitation light induces cell damage (phototoxicity caused by the generation of free oxygen radicals). Therefore, the intensity and dose of excitation light needs to be minimized in order to get unbiased data from live samples. On the other hand, less excitation light means fewer photons will be emitted by the fluorescent label and the detector will struggle with a low SNR.

Expression levels of fluorescent labels

To make things worse, expression levels of exogenous recombinant proteins in cell assays should be kept low to avoid artifacts from overexpression. For example, viral transfection of vectors in effect adds populations of additional molecules to the pool of endogenous molecules of interest, potentially disturbing the “natural” balance and ultimately leading to biased results.

Genome editing technologies (CRISPR / Cas9 system, TALEN, ZFN; Nature Methods’ Method of the Year 2011, [1]) have recently been developed to address this issue. They allow the interrogation of virtually any protein or set of proteins at endogenous expression levels. This poses an additional challenge for imaging systems as signal levels can be very low. A quick calculation shows that, at 10 nM protein concentration, only 6 fluorescent molecules will be found in the typical confocal detection volume of 1 fL.

Small Structures

Cellular machinery is organized in cellular subcompartments with sizes in the sub-micrometer to nanometer range. Decoding this machinery and watching it at work requires an imaging system with sufficient spatial and temporal resolution. For practical reasons the resolution of conventional CLSMs is limited to about 200 nm or more in X and Y, and 700nm in Z, respectively.

Thus the ideal next generation CLSM would need to provide a balanced and flexible combination of:

- image quality (SNR) to identify and localize biological molecules of interest while minimizing input of light,
- speed to monitor these molecules and their interactions in space and time, and
- high resolution to image intracellular structures with sufficient detail in X-Y-Z.

Image scanning with a segmented detector array

An acentric, shifted pinhole detector produces an image of about the same resolution as a pinhole detector which is aligned to the optical axis, although smaller in amplitude and shifted by half the displacement. This insight has been the motivation for constructing an area detector for a confocal microscope. Such a detector should cover more than 1 AU and contain multiple sub-Airy detector elements. Detection efficiency will be significantly increased by reassigning the detected photons from the shifted detector elements to the central detection position and summing up the back shifted signal from all detector elements. [2] No light is rejected by a closed pinhole but instead collected by the off-axis detector elements. Therefore an increased signal level arises from the reassignment of photons to a smaller spatial region. [3] At the same time this method delivers a lateral resolution enhancement by a factor of 1.7. [4, 5, 6, 7]

Airyscanning delivers enhanced resolution in all three spatial directions

Pure reassignment of detection does not improve axial resolution. [5] However, each detector element also acts as an individual pinhole. Each image can be individually deconvolved and weighted, and photons can be precisely reassigned. The additional information can be used for an isotropic, 1.7 fold-increase in resolution in all spatial directions (Figure 1).

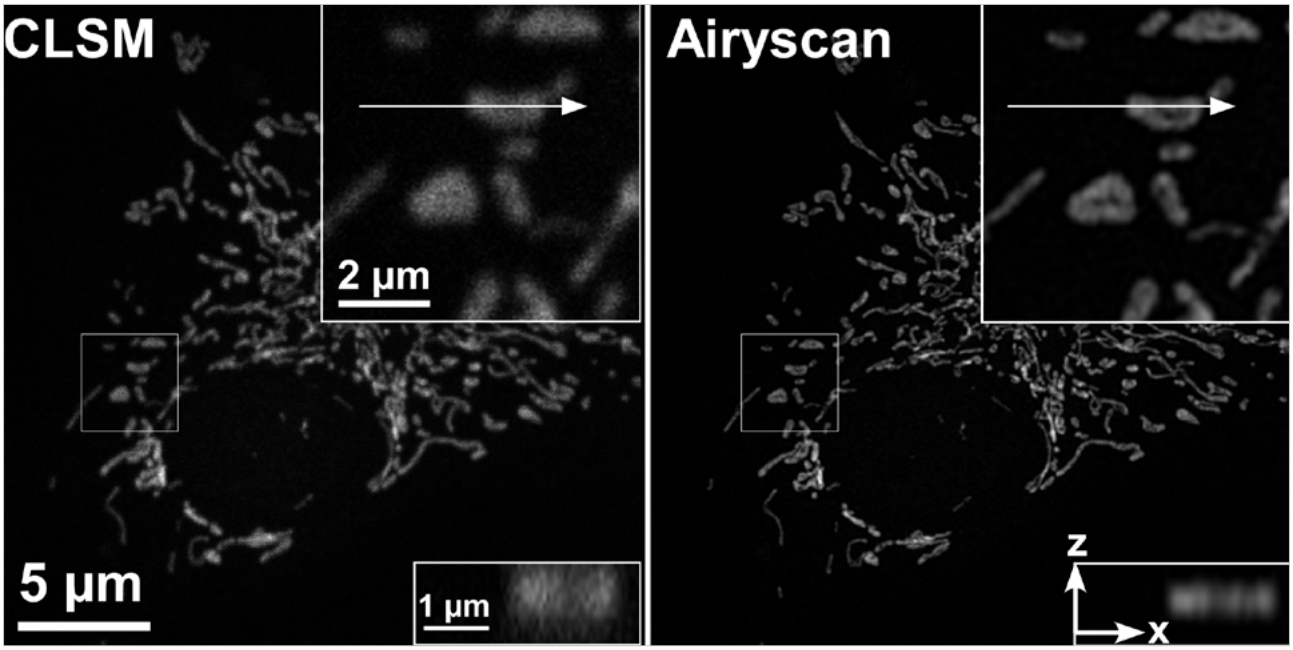

Figure 1 *Confocal (CLSM) and Airyscan recording of a FluoCell #1 (InVitroGen) imaged for MitoTracker to visualize mitochondria. Shown in the upper right corner is a magnified view of the area indicated by the smaller squared box. The lower right corner shows an xz section along the indicated arrow in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

Conclusion

Airyscanning releases the full potential of a confocal microscope, achieving resolutions comparable to an extremely small pinhole but with a much better signal-to-noise ratio. Higher SNRs enable higher acquisition speeds, thus allowing to image fast processes in 3D. Airyscanning works for any dye and is especially suitable for live cell imaging as the high sensitivity of the detection system allows to apply lower laser powers.

Airyscanning is based on confocal point scanning. Even thick samples can be penetrated easily in a situation where widefield-based superresolution techniques would struggle. Airyscanning lets the users decide whether to gain 1.7x higher resolution in all three dimensions, resulting in a 5x smaller detection volume. Or to push sensitivity beyond the limits of conventional confocals. Or to use the increase in signal-to-noise ratio to speed up image acquisition. It's all about choice.

References:

[1] Nat Methods, 2012. 9 (1)

[2] Sheppard, C.J., Super-resolution in confocal imaging. Optik, 1988. 80(2): p. 53-54.

[3] Sheppard, C.J., S.B. Mehta, and R. Heintzmann, Superresolution by image scanning microscopy using pixel reassignment. Opt Lett, 2013. 38(15): p. 2889-2892.

[4] Muller, C.B. and J. Enderlein, Image scanning microscopy. Phys Rev Lett, 2010. 104(19): p. 198101.

[5] York, A.G., et al., Resolution doubling in live, multicellular organisms via multifocal structured illumination microscopy. Nat Methods, 2012. 9(7): p. 749-754.

[6] Roth, S., et al., Optical photon reassignment microscopy (OPRA). Optical Nanoscopy, 2013. 2(5): p. 1-6.

[7] York, A.G., et al., Instant super-resolution imaging in live cells and embryos via analog image processing. Nat Methods, 2013. 10(11): p. 1122-1126.

Cover Image Credits:

Left panel: State-of-the-art confocal detection

Right panel: Airyscan detection

Top: Fixed tumor cells, tubulin labelled with Alexa 555. Sample: courtesy of P. O`Toole and P. Pryor, University of York, UK

Middle: HeLa cells, Actin stained with Phalloidin-Alexa 546, AP3 with Alexa 488, DAPI. Courtesy of S. Traikov, BIOTEC, TU Dresden, Germany

Bottom: FISH on chromosomes from IMR90 fibroblasts; PNA probes labelling telomeric G- (Alexa 488, green) and C-strands (Alexa 546, red), resp.; DNA (DAPI, blue). Sample: courtesy of J. Karlseder, Molecular and Cell Biology Laboratory, and J. Fitzpatrick, Waitt Advanced Biophotonics Core, The Salk Institute, La Jolla, USA.

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