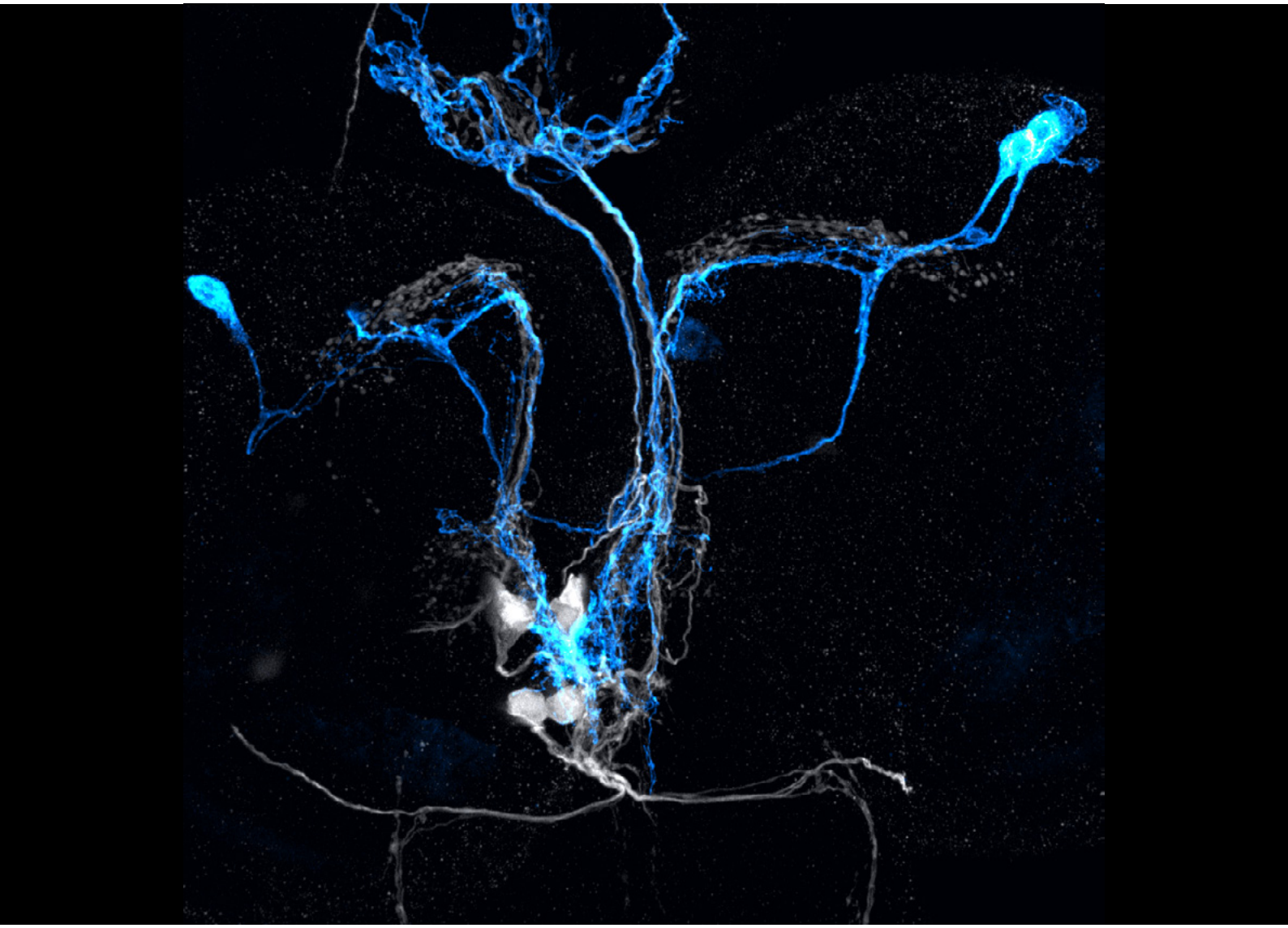

Quick Insight

Improving Resolution, Sensitivity and Speed of Your Confocal Imaging

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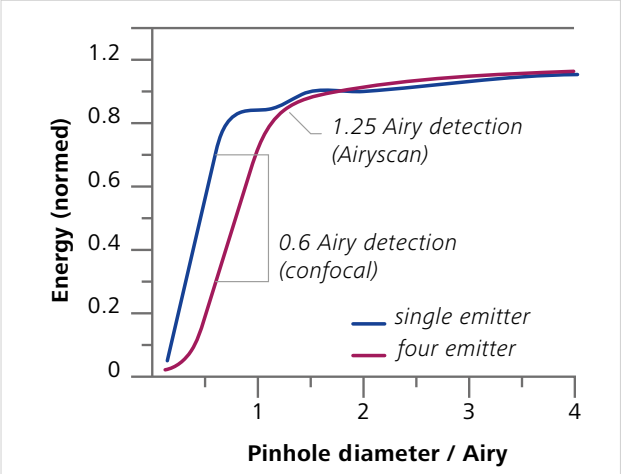
Why is this important for my imaging?

Fluorescent microscopy has become a routine task in biomedical research, and there is a great chance that it is done with a confocal laser scanning microscope (LSM). With their highly desirable ability to produce optical sections instantly and with very high contrast, they are popular microscopes for a variety of imaging tasks [1].

By placing an aperture, the pinhole, in the emission light path an LSM blocks out of focus fluorescence signal resulting in the ability to create an optical sectioned image. Additionally, the resulting image resolution, and therefore the ability to tell structures apart, benefits from closing the pinhole more and more. But everyday life teaches us that if closing the entry point of light, for example closing our eyes, it gets dark very quickly, as is the same for LSM imaging (graph 1). Generally speaking, the more photons the microscope system is able to detect, the better the resulting image in terms of signal to noise ratio (SNR). So for the past decades, the pinhole has been set to a good compromise for resolution and available light (1 airy unit, graph 1).



Graph 1 *The graph shows how much light can pass through a pinhole in dependence of its opening diameter. The case for a single emitter, which would be a single fluorophore molecule is depicted in blue. The case of four emitting fluorophores, which represents closer the real live situation of a labelled sample, is shown in red. The energy available for 0.6 AU, used in a confocal, and 1.25 AU used routinely in Airyscan are indicated by arrows. When closing the pinhole from 1.25 AU to 0.6 AU, about 60 – 70 % of the emission light is lost for a fluorescently labelled sample [2, Box 4]. The graph is based on simulations of the available energy, i.e. the number of photons, for imaging in dependence of the pinhole diameter size (in AU). The Energy was normed to the value at 1 AU of the four emitter case.*

Since 2009 one development to improve SNR on an LSM was to enhance the photon gathering ability of the system by changing the photocathode material of a PMT detector. High-end technology like the GaAsP cathode material, made the traditional PMT detector up to 2.0 × more sensitive to photons. Naturally GaAsP cathodes are used nowadays in many different detector types (e.g. GaAsP PMT; Hybrid detectors) of most of the commercially available LSM.

However, if too little emission light is available, one countermeasure to increase the SNR is to enhance the excitation laser power, which will create more photons but likely destroy the needed fluorescent label by bleaching, resulting in a vicious cycle of reducing the fluorescent signal even more. Furthermore this will damage living samples at the same time due to phototoxicity [3, 4]. The other option for higher SNR is longer exposure times to allow for more photons to be collected, compromising on the achievable acquisition speed and again increasing the overall light dose on the sample. A better option especially for live cell imaging would be to increase the efficiency of detecting photons.

So it is paramount to gather the highest amount of photons, resulting in an excellent signal to noise ratio (SNR) which is the basis for resolution, speed and sensitivity in LSM imaging experiments.

Deconvolution

To improve the resolution of LSM images, deconvolution (DCV) processing has long been a possibility. DCV has been an integral part in fluorescent widefield imaging for many years, resulting in optical sections. DCV works universally on images acquired with different types of fluorescent microscopes. This advantage is utilized by including all necessary information for DCV within the image's metadata, so the variety of experimental data from e.g. widefield, LSM, or multiphoton systems is automatically recognized and processed accordingly by ZEN software. Recently, new GPU-based technology allows for dramatic increases to the DCV processing speed by a factor of up to 20 – fold, reducing the computation time of DCV. This gain in processing performance makes excellent use of available sophisticated algorithms for best results.

The principle of deconvolution is to calculate the location from which the detected photons originated from. The more photons are available, hence the higher the SNR in the original data, the better the processing results in terms of achievable resolution.

This holds true when DCV processing is used with LSM images. However, to obtain super-resolution the pinhole of the LSM needs to be closed to about 0.6 airy units (Fig. 1, dark blue circle) to improve the optical resolution performance. Hence, DCV with LSM images are an option if the inevitable light-reduction is acceptable for the sample and can be compensated by a combination of longer exposure times, averaging, or higher laser powers to generate the necessary SNR. Consequently, this combination has limited suitability for living samples or generally low endogenous expression levels (graph 1).

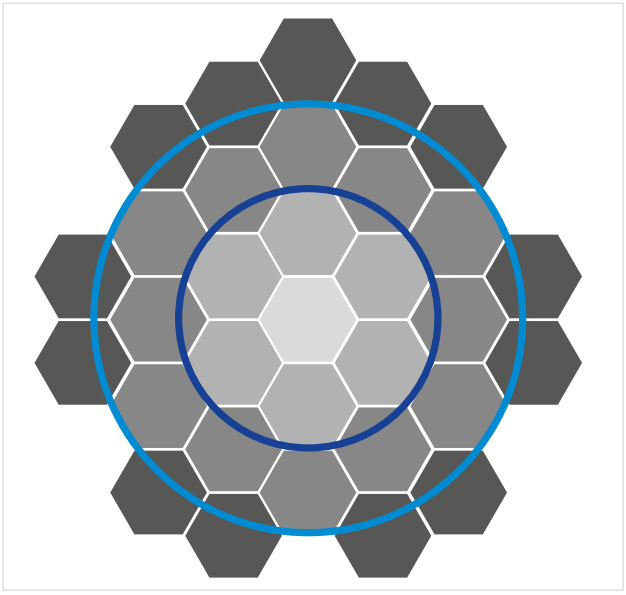


Figure 1 *This shows the area of the Airyscan detector with its 32 detector elements. The complete area is used to capture light (1.25 AU). The cyan circle matches the area to capture light with conventional confocal settings (1 AU), and the dark blue circle equals the area to capture light for LSM with DCV to achieve a similar resolution than Airyscan (0.6 AU). Closing a pinhole from 1.25 to 0.6 AU means efficiently a loss of about 60 – 70 % of light from the sample. [2]*

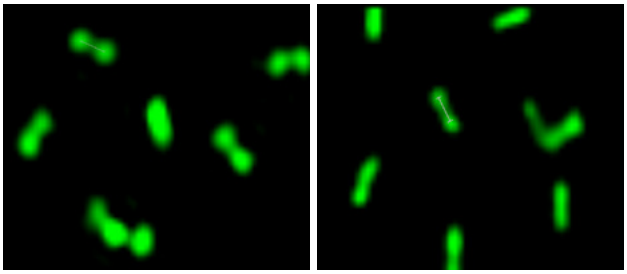


Figure 2 *Airyscan (left image) and LSM (GaAsP detector) with DCV (right image) resolve the labeled ends of nanorulers [6] with a defined distance of 140 nm. For both images emission range (500 – 550 nm), laserpower (0,2 % of 488 nm), pixel dwell time (16 µs) and sampling (256 × 256 pixels) are the same. Dissimilar settings were necessary for averaging and z-slices.*

	Airyscan	LSM + DCV
Average	none	4
z-slices	1	14

In order to perform DCV for superresolution, information of several z-slices needs to be collected. Furthermore the loss of light at the smaller pinhole opening is compensated by repeated imaging of one frame to collect more photons (averaging). In this example acquisition time and laser exposure are by a factor of 56 (4 × 14) lower for Airyscan. This makes Airyscan imaging the faster choice for superresolution imaging.

LSM with DCV, with or without GPU, will provide:

- ⊕ Enhanced resolution
- ⊕ Enhanced contrast
- ⊖ No enhanced light collection, instead light reduction for super-resolution imaging
- ⊖ Needs the acquisition of at least 3 to 5 z-slices for true resolution increase

in combination with:

- ⊕ GPU for faster data processing
- ⊕ All LSM lasers (405 – 639 nm) and multiphoton excitation
- ⊕ All fluorescent labels
- ⊕ All LSM imaging modes, including spectral lambda scans for up to 10 different labels
- ⊖ Thin samples (best suitable to about 30 µm)[1]

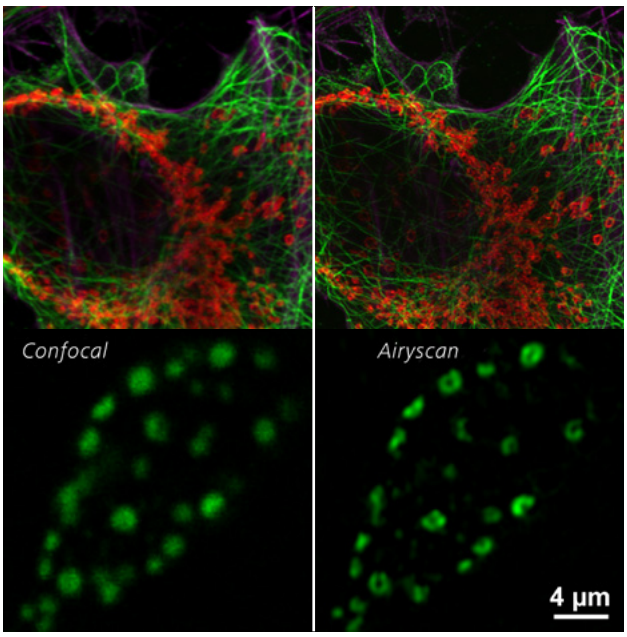


Figure 3 *These examples show the enhanced resolution and signal to noise of images taken with Airyscan (right side) compared to images taken with traditional LSM GaAsP detectors (left side). HeLa Cells with triple labelling (red, green purple),upper panel, and Drosophila neuromuscular junction stained for Bruchpilot (BRP), lower panel (J. Pielage, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland).*

Airyscan and Fast Acquisition Mode

Airyscan is an innovative detector that enhances the ability of the LSM system to collect more photons along with increased spatial information. This surplus in SNR and spatial information is simultaneously improving sensitivity, resolution and speed of the LSM system – the most extreme step to improve LSM imaging in the past decade. Instead of a single detector, 32 GaAsP detectors form an area to capture fluorescent light (Fig. 1).

Each of the 32 detectors on its own acts like a detector behind a closed pinhole, increasing the optical resolution of the system but collecting only a limited amount of light (graph 1). With 32 detectors the situation changes dramatically. Now significantly more photons are captured than with the conventional 1 airy unit pinhole opening (Fig. 1), and about 3.6 times more than with a closed pinhole of 0.6 AU [2]. With this SNR advance, dim and sensitive samples are captured with improved resolution, allowing for easy and gentle

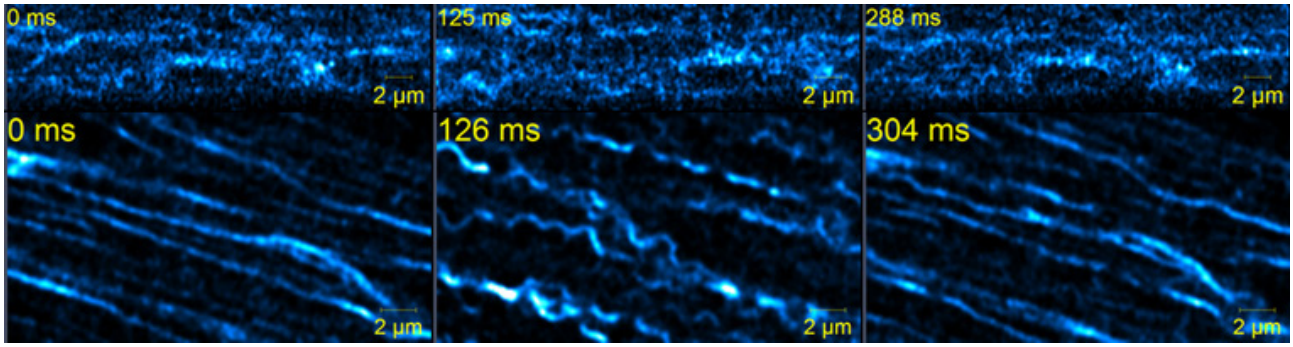


Figure 4 *The comparison of high speed imaging, here shown with still pictures of time series experiments, with the resonance scanner (top portion) and Airyscan in Fast mode (bottom portion) shows the difference in obtainable data. While the structural information is lost in resonant scanning, it is clearly visible in Airyscan Fast mode acquisition. Airyscan in Fast mode allows for efficient photon collection at high speeds. Cardiomyocyte Cells with tubulin-EMTB to measure microtubule buckling. Top images: Resonance Scanner using standard confocal, Acquisition speed: 80 fps. Bottom images: LSM 880 Airyscan Fast, Acquisition speed: 96 fps, movie: https://youtu.be/TOPpeNdqvJI. Images and Samples courtesy of Ben Prosser, University of Pennsylvania, USA [5]*

[1] *Dependent on light absorption and scattering of the sample*

super-resolution imaging, and even allowing a reduction of the potentially harmful illumination laser to powers well below 1 % (Fig. 2, 3). For extremely dim samples, the Airyscan detector can operate in an even more sensitive mode, collecting photons from 2 AU pinhole opening, making for example CRISPR / Cas9-mediated endogenous protein imaging possible and efficient. Pushing the abilities of the Airyscan detector even further, it combines outstanding photon collection with enhanced imaging speed and resolution with its Fast Mode. Instead of imaging only one line at a time at high speed as it is the principle of resonant scanning, and leaving only little time to capture light, Airyscan uniquely images four lines at once. Consequently the image is taken at a faster speed while more time is spent to collect photons from each image point, making it the most efficient scanning method today. This makes Airyscan with Fast the ideal choice to image dynamic processes in living samples or capture large volumes without compromising image quality or resolution (Fig. 3).

When performing LSM imaging, Airyscan and Fast acquisition mode will give you:

- ⊕ Enhanced light collection = enhanced signal to noise (SNR)
- ⊕ Up to 4 – 8 × higher sensitivity of the system
- ⊕ Up to 1.7 × better resolution in all dimensions (x, y, z)
- ⊕ Up to 4 × enhanced imaging speed
- ⊕ Works for single slices (2D) and z-stacks (3D) imaging

in combination with:

- ⊕ All LSM lasers (405 – 639 nm) and multiphoton excitation
- ⊕ All fluorescent labels
- ⊕ Thick samples (> 100 µm) with robust and reliable results
- ⊖ Not combinable with spectral lambda scans

Summary

Airyscan and DCV (with or without GPU) are options to obtain superresolution images of your samples with a confocal LSM. But only Airyscan and its Fast acquisition mode offer additional benefits of collecting more light from your sample; which allows for fast and sensitive super-resolution imaging (Fig. 2).

References:

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[6] GATTA-SIM 140B, distance=140 nm, Alexa 488. GATTAquant DNA Nanotechnologies, www.qattaquant.com

Title:

Drosophila larval brain, Hugin neurons (white, labelled by YFP) and neurons expressing the neuropeptide corazonin (blue, labelled by mRFP), acquired with LSM 880 Airyscan. Image provided by Dr. S. Hückesfeld, Live & medical Sciences Institut (LIMES), Pankratz Lab, Molecular Brain Physiology and Behavior

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