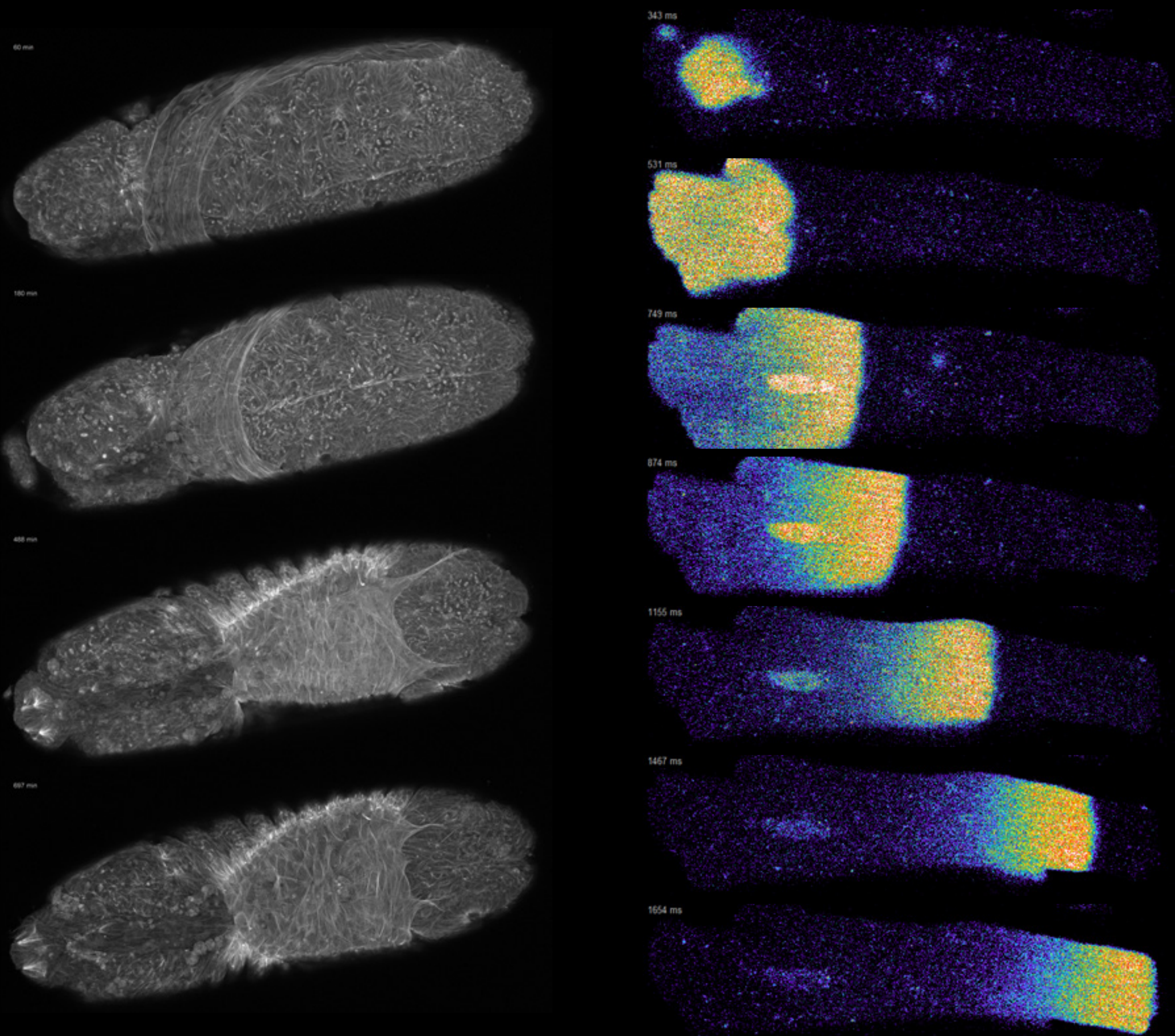
Technology Note



ZEISS LSM 880 with Airyscan

Introducing the Fast Acquisition Mode

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In August 2014, ZEISS introduced Airyscan, a new detector concept for confocal laser scanning microscopy (LSM). Airyscan is a 32 channel GaAsP-PMT area detector, positioned at the pinhole-plane of an LSM. Using Airyscan, additional light and spatial information is collected beyond that of a typical LSM image, resulting in substantial and simultaneous improvements in spatial resolution and signal-to-noise ratio. The introduction of the Fast mode for Airyscan represents the next innovation step for LSM imaging. Airyscan detector technology is utilized along with an illumination shaping approach to enhance acquisition speeds by four times. Airyscan affords researchers access to superresolution, increased signal-to-noise ratio and increased acquisition speeds simultaneously without the traditional compromises.

Laser Scanning Microscopy

The Confocal Laser Scanning Microscope (LSM) has become one of the most popular instruments in basic biomedical research for fluorescence imaging. The main reason LSM has become so popular is that the technique affords researchers images with high contrast and a versatile optical sectioning capability to investigate three dimensional biological structures [1]. The optical sectioning ability of an LSM is a product of scanning a diffraction limited spot, produced by a focused laser spot, across a sample to create an image one point at a time. The generated fluorescence from each point is collected by the imaging objective and results from fluorophores in the sample that reside both in the desired plane of focus and in out of focus planes. In order to separate the fluorescence emitted from the desired focal plane, an aperture (pinhole) is positioned in the light path to block all out of focus light from reaching the detector (traditionally a PMT) [2]. Based on the application needs, LSM offers tremendous flexibility to fit experimental requirements, such as the choice of the excitation laser wavelengths and scanner movement; magnification and resolution of objective lenses as well as the type and arrangement of the detectors. Hence LSMs can be used to image diverse samples from whole organisms to large tissue sections to single cells and their compartments, labeled with numerous fluorescent markers of diverse emission intensities. During the past couple of decades the LSM has undergone continuous improvement; both usability and technical capability of the instruments (to make use of the precious emission light) have been significantly enhanced. These improvements have been the result of constant technical advances, production of high class optical components and improvements in the design of the confocal beam path. But the one ultimate compromise of confocal laser scanning microscopy was not touched until 2014, when ZEISS introduced the Airyscan for its LSM 8 Family systems: the pinhole.

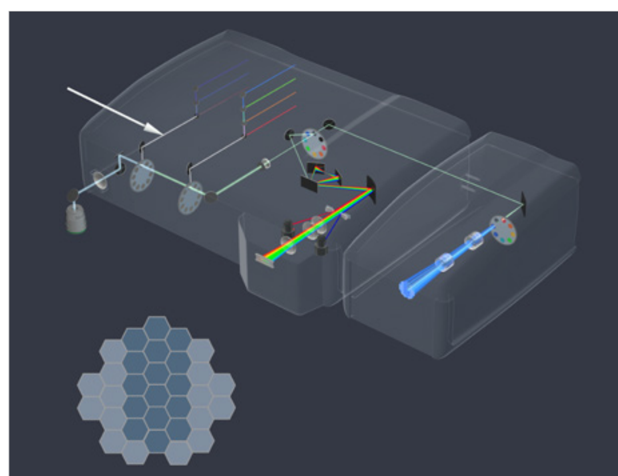

Figure 1 *LSM 880 with Airyscan beam path. For Fast mode imaging, the wheels holding the slit apertures are introduced into the illumination beam path (arrow), shaping the excitation beam into an ellipse. The emission light is captured on the 16 center detector elements (grey) of the Airyscan detector. The remaining 16 detector elements are not used in Fast mode imaging. The Airyscan detector itself remains unchanged and all 32 detector elements are used for Airyscan modes (e.g. superresolution or sensitivity mode).*

Until this point the pinhole would be generally set to a 1 Airy unit (AU) opening diameter; resulting in a good compromise between capturing the scarce emission light and achieving an effective resolution. In theory one can enhance the resolution of a confocal LSM by closing the pinhole below a 1 AU opening. However this is not usually an option, since too much light is rejected resulting in images with unusable signal-to-noise (SNR) ratios. For the first time, the Airyscan detector allowed to combine enhanced resolution and signal to noise for LSM imaging [3].

Airyscan detector

The Airyscan detector consists of 32 GaAsP PMT detector elements, which are arranged in a hexagonal array (Figure 1), positioned at a conjugated focal plane in the beam path the detector is functioning as the traditional LSM pinhole.
For full flexibility an adjustable optical zoom is present in front of the Airyscan detector which enables adjustment of the number of Airy units that are projected onto the detector.

This design made it possible to collect more light (equivalent to a pinhole opened to 1.25 AU), whilst at the same time dramatically enhancing the resolution, with every detector element acting as an efficient pinhole with a diameter of only 0.2 AU. Instead of facing an either / or decision, a simultaneous enhancement of resolution by the factor of 1.7 x and signal-to-noise by 4 – 8x was introduced to LSM imaging. Superresolution imaging under gentle conditions, with low laser powers, became part of the confocal LSM repertoire. Flexibility was added with the zoom optic, which allowed researchers to decide if resolution or sensitivity was the priority for the experiment; adapting the Airyscan advantages to the specific experimental needs. Using either multiphoton or single photon excitation without altering the well-established LSM sample preparation and labelling protocols, further broadened the experimental prospects.
Detailed descriptions of the theory and technology of Airyscanning can be found in these technology notes [4, 5].

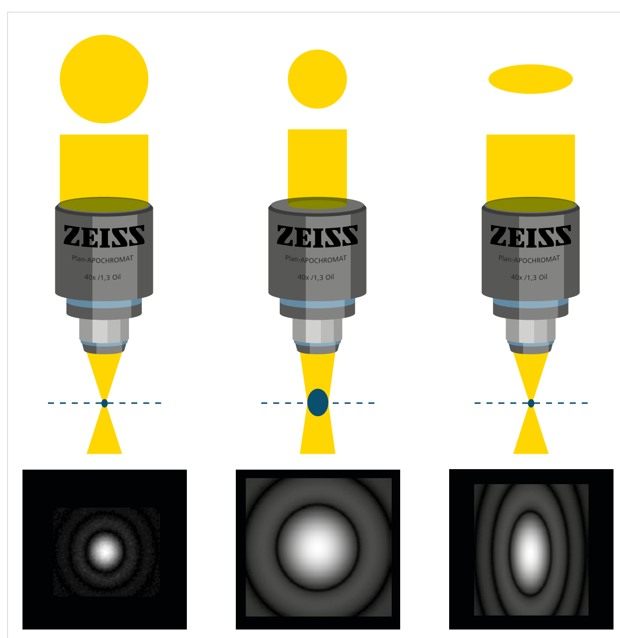

Figure 2 *The shape of the laser beam, that enters the back aperture of the objective lens, determines the resulting excitation PSF. Conventionally it is the goal to generate the smallest possible excitation spot with a given objective lens. This is achieved with a laser beam that completely fills the back aperture of the objective lens (left). If the laser beam's diameter is smaller than the back aperture of the objective, the resulting PSF is larger (middle). In order to elongate the excitation PSF only in one direction, an elliptical laser beam is used. This beam is narrowed along one axis, stretching the resulting excitation PSF along that exact axis (right).*

Limitations of acquisition speed in conventional LSM

Research objectives can dictate the acquisition of fast, dynamic processes or the quick capture of many fields-of-view (FOV). In both cases, the challenge for the imaging system is to collect sufficient fluorescence for an image with good SNR but in a very limited period of time.
Conversely, because traditional LSMs create images one point at a time, image acquisition can be relatively slow.
To improve the acquisition speed of LSM instruments, several strategies can be pursued; such as limiting the field of view, sacrificing image resolution (using fewer image pixels) and scanning the laser spot faster.
When scanning the laser spot faster across a FOV, the pixel dwell time is shortened. Consequently, the amount of time per pixel spent collecting fluorescence is also shorted which impacts the resulting SNR of the image. As the acquisition speed is increased, fewer and fewer photons will be available resulting in a deterioration of image SNR. The outcome is not only a noisy image but also a compromised spatial resolution, in which fine structures cannot be properly resolved. To compensate for the deteriorating SNR the laser power can be increased but this too has disadvantages; the danger of bleaching the fluorophore and / or damaging live samples by phototoxic effects (e.g. free oxygen radicals) becomes more prevalent at higher laser powers and thus the risk of influencing experimental outcomes is increased [6, 7, 8,].
Therefore, traditional techniques to improve image acquisition speeds demand that a researcher compromises image SNR, resolution, FOV and laser exposure, all of which will likely impede the research goal.

Fast mode

To solve the traditional trade-off between acquisition speed and image SNR, the Airyscan detector is used in a new Fast acquisition mode. As an area detector the Airyscan can capture spatial information that is utilized to parallelize the scanning process, collecting 4 image lines simultaneously. This means enhancing acquisition speed by a factor of 4 while keeping high pixel dwell times to efficiently collect emitted photons. Ordinarily, the focused laser beam is moved along the x-axis to acquire one image line, before it is moved in the y-axis to acquire the consecutive image line. In Fast mode imaging, four image lines are acquired at the same time when moving the laser in the x-direction.

In order to excite the fluorescent dye in four lines at a time, the excitation spot needs to be broadened slightly along the y-axis. The broadening is achieved by shaping the laser beam before it enters the objective lens back aperture (Figure 2). If the laser beam is narrowed in its y-axis before entering the objective lens, the resulting excitation beam is stretched into an ellipse along the y-axis, while its size in x direction remains unchanged. In LSM 880 the beam shaping is performed by using slit apertures positioned in the excitation path of the scanhead. Different slit sizes are provided to serve a wide variety of objective lenses. These slit apertures are therefore arranged on wheels that position the necessary slit width into the laser beam path (Figure 1). The excitation ellipse is scanned along the x-axis of the image field in the conventional manner; but at the end of each line, the laser beam is shifted by the distance of 4 pixels in y direction before scanning the next line. The imaging time for one frame is thus reduced 4-fold without reducing the pixel dwell time in the process.

The resulting fluorescence for each 4-pixel column is collected by the Airyscan utilizing 16 detector elements of the Airyscan detector's center (Figure 3) where three horizontal detector elements cover 0.9 AU and the up to 6 vertical elements cover 1.65 AU of the emission Airy disk[1]. As a result, each detector element acts as an individual pinhole with a diameter of about 0.3 AU.

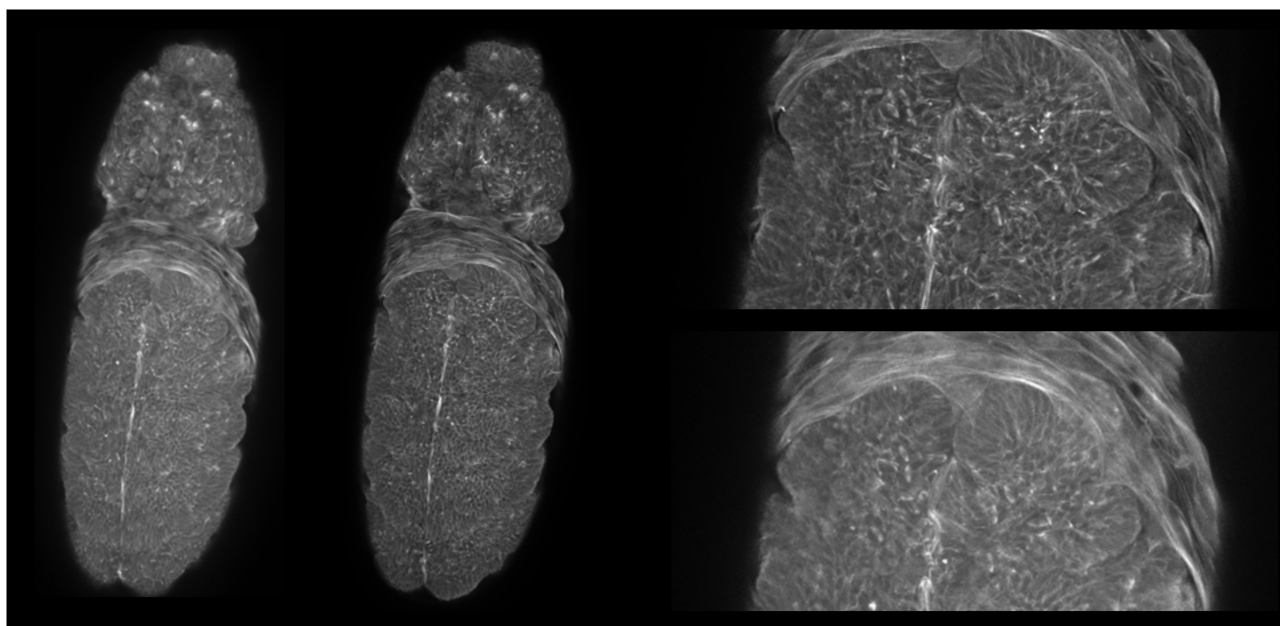

Figure 3 *Drosophila melanogaster embryo, Jupiter-GFP (microtubules). The left hand image was acquired with the internal GaAsP detectors of LSM 880. The z-stack (80 images) acquisition took 4:47 minutes. The same z-stack was acquired afterwards in Fast mode imaging in only 1:11 minutes. The comparison (close-up; upper image: Fast mode, lower image: internal GaAsP detectors) shows, that as well image quality in Fast mode is superior to the conventional confocal image.*
Settings for both images: Optimal sampling: 3372 x 1451pixels; Plan-APOCHROMAT 20x / 0.8; Z-stack: 80; Pixel dwell: 0.62 µs
Sample courtesy of B. Erdi, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, Austria.

1 For image pixel sizes that correspond to at least Nyquist sampling or superresolution sampling; named Optimal and SR sampling in ZEN.

The remaining 16 detector elements, of the otherwise unchanged Airyscan detector, are not used and do not produce any digital data. This keeps the data rate lean when streaming it directly onto the hard drive. Each individual detector element of the Airyscan detector is shifted relative to the optical axis by a certain distance. Therefore the captured signal must be reassigned to its point of origin within the resulting image. Consequently emitted photons are not rejected at a pinhole aperture but are rather collected and contribute to the signal of the respective pixel to increase its intensity. This pixel reassignment process, performed on the mathematical basis published by Sheppard et al. [9,10], results in the 4 vertical pixels per laser beam position.
The resulting image from the Airyscan in Fast mode shows an enhanced SNR and resolution, because the detector collects more light than with conventional LSM settings, and it combines this with the resolution of a very small pinhole. The concluding deconvolution step therefore profits from both a very small effective PSF and a high SNR.
As for conventional point scanning LSM, Fast mode works reliably in thicker samples; and can be used with multiphoton excitation to analyze highly scattering tissue.

Conclusion

The introduction of Airyscan eliminated the requirement to choose between high resolution and high sensitivity; both could be achieved at once. In the same way, Airyscan Fast mode now takes this one step further by enabling simultaneous improvements in resolution, sensitivity and speed.

Using Airyscan in Fast mode enables the use of this unique GaAsP area detector for spatial parallelization to enhance imaging speed without compromising pixel dwell time. Airyscan in Fast mode delivers images with 4 times more SNR at a 4 times increase in acquisition speed. At the same time the characteristic advantages of the Airyscan are preserved and allow for increased resolution by a factor of 1.5 x. Furthermore, these advantages can be realized without making any changes to sample preparation or staining protocols and can be seamlessly integrated into current experimental workflows.
The result of simultaneously improving resolution, SNR and speed on an optical sectioning system provides researchers with the unique combination of gentle imaging with high spatial and temporal resolution. This unprecedented combination of functionality promises to meet the growing demand for efficient large volume imaging whilst also addressing large scale structural studies and providing the capability of capturing dynamic processes for functional analysis.
With Fast mode for Airyscan, ZEISS expands the potential of the Confocal Laser Scanning Microscope.

Fast mode characteristics		
Fast mode	LSM 880 with Airyscan acquisition mode to acquire 4 image lines simultaneously, increasing image acquisition by 4-fold	
Airyscan detector in Fast mode	16 central detector elements of the Airyscan detector are active. The remaining 16 detector elements are not used for Fast mode acquisition.	
AU per element	~ 0.3 AU	
Resolution	Enhanced by 1.5 fold	
	x = 145 nm, y = 180 nm, z = 450 nm	
Sensitivity	4 x enhanced SNR at 4 times faster image acquisition	
Speed	512 x 512 pixel	19 fps
	480 x 480 pixel	27.3 fps
	480 x 128 pixel	86.1 fps
	1024 x 1024 pixel	6.2 fps
	2048 x 2048 pixel	1.6 fps

Glossary	
Airy disk	The center spot of the Airy pattern.
Airy pattern	A single point source is imaged by a microscope as a blurred spot with surrounding rings of decreasing intensities, due to the diffraction nature of light.
Airy Unit (AU)	Diameter of the Airy disk, measured from the first surrounding intensity minimum.
GaAsP	Gallium arsenide phosphide. Semiconductor material, which is used as a coating for the photocathode of the detector. The photocathode converts photons into electrons.
LSM	Laser scanning microscope
Pinhole	Aperture, positioned in the conjugated focal plane in the emission beam path, blocking out-of-focus light.
Pixel dwell	Duration the laser is illumination one position and the microscope system is collecting emission light, to generate one image pixel
PMT	Photomultiplier tube; common basis for light detectors in Laser Scanning Microscopes
PSF	Point spread function. Describes the pattern that is generated by a microscope of a point emitting light source.
SNR	Signal to noise ratio.

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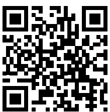
Title:

Left side: Single images of a time series. Calcium sparks labeled with Fluo 4 imaged in Cardiomyocytes with 50 frames per second. Courtesy of P. Robison, B. Prosser, University of Pennsylvania, USA

Right side: Single images of a time series. Drosophila embryo, maximum intensity projection. Microtubules labeled with GFP. Z-stack with 72 slices imaged for 11.5 h at 15 min interval. Courtesy of B. Erdi, Max F. Perutz Laboratories, University of Vienna, Austria



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