ZEISS

Multiplex mode for the LSM 9 series with Airyscan 2: fast and gentle confocal super-resolution in large volumes

The LSM 9 family with Airyscan 2 from ZEISS provides more options to enable the perfect balance of speed and resolution for today's confocal-imaging needs. By combining the pinhole-plane imaging concept of Airyscan 2 with improved illumination and detector readout routines, the new Multiplex mode extracts more spatial information, enabling parallel pixel reconstruction. Hence, multiple lines can be imaged in a single line scan, which allows for bigger acquisition steps to improve image-acquisition speeds and reduce the illumination dosage to the sample.

The LSM 9 series with Airyscan 2 represents the next big step in the evolution of confocal microscopy. With a focus on usability, utility, and throughput, the Airyscan 2 detector from ZEISS fully leverages pinhole-plane imaging with new data-handling and acquisition strategies. The net result of these innovation steps is not only an improved user experience but the new Multiplex mode. Through new acquisition strategies for pinhole-plane imaging, the new mode empowers researchers to leverage the unique combination of optically sectioned super-resolution and sensitivity at the highest volume rates. The Multiplex mode innovation for Airyscan 2 focuses on the need to capture structural dynamics, cellular signaling, molecular trafficking, and diffusion events with real-time super-resolution and superior signal-to-noise ratio (SNR) in model systems that extend beyond traditional cultured cell preparations and into new 3D model systems of organoids, spheroids or 3D culture, and whole organisms.

The new Multiplex mode

The use of a physical aperture (i.e., pinhole) and unitary detector (standard photomultiplier tube (PMT)) in the design of traditional laser-scanning microscopy systems requires that the final data resolution, SNR, and speed be directly coupled to how finely an image is scanned and how the data are read off the PMT (Fig. 1). The traditional design yields a 1:1 relationship between the scanner movement and the data readout in which every scan position (i.e., pixel) correlates to one value read from the unitary detector (i.e., pixel intensity). Hence, the scanner–detector relationship directly affects what model systems, experiments, and applications can be used with a laser-scanning system, as researchers have traditionally been forced to compromise between scanning speed (i.e., frame rate), pixel dwell time (i.e., SNR and laser exposure), and pixel size (image resolution). The interplay and codependence that were once leveraged as experimental flexibility have now become the limiting factors for traditional confocal systems. With model systems and organisms getting larger and with lower expression levels (e.g., lower fluorophore concentrations), traditional confocal systems are severely restricted in terms of how quickly desired sample volumes can be scanned with sufficient SNR and resolution, and this ultimately affects the ability to quantify the image data.

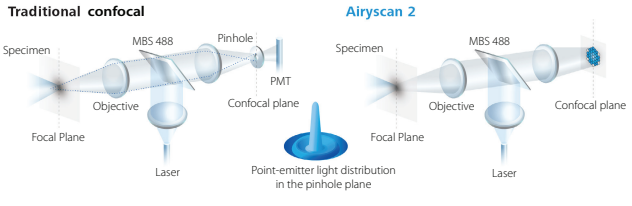

Figure 1 |Comparison of traditional confocal and Airyscan 2 beampaths. Left, a schematic of a traditional laser-scanning confocal microscope with a pinhole–PMT arrangement. Right, a schematic showing the placement of the Airyscan 2 detector in the pinhole plane, acting as a series of small 0.2–Airy Unit (AU) pinholes to provide improved optical resolution with the collection ability of a 1.25-AU pinhole. MBS 488, main beam splitter used with a 488-nm laser.

The innovative design of the ZEISS Airyscan 2 detector enables the expansion of the scanner–detector codependence beyond the traditional 1:1 relationship by fully leveraging the positional information contained at the pinhole plane. The power of the new Multiplex mode arises from the fact that both the structure of the excitation laser and the structure of the detection are leveraged to parallelize image acquisition while still providing simultaneous higher SNR and super-resolution (Fig. 2). The Airyscan 2 detector always captures a detailed image of the pinhole plane, allowing the excitation laser to be stepped farther and more coarsely over the field of view to improve the acquisition speed. Because of the high amount of spatial information captured in the pinhole plane image, a final image is reconstituted with finer resolution than in acquisition

Joseph Huff*, Annette Bergter and Benedikt Luebbers

Carl Zeiss Microscopy GmbH, Jena, Germany. *e-mail: joseph.huff@zeiss.com

sampling. Therefore, with Multiplex mode for Airyscan 2, the final image resolution is dictated by the detection sampling and not the excitation sampling. By changing the acquisition sampling steps to match the inherent oversampling of the pinhole-plane imaging, the user can parallelize the image acquisition from 2× to up to eightfold while still achieving super-resolution imaging with superior SNR at speed (Fig. 2).

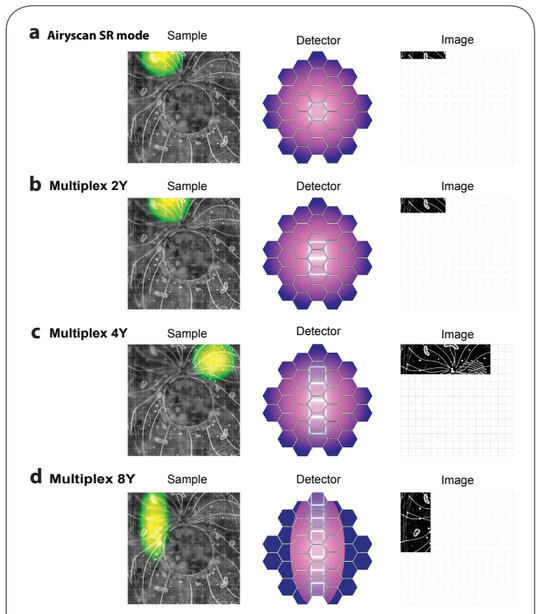

Figure 2 | Depictions of the image-acquisition schemes available on the new Airyscan 2. **a**, Standard Airyscan SR (super-resolution) imaging. **b**, Available on LSM 900, the Multiplex 2Y mode acquires two image pixels in parallel. **c**, Available on both LSM 900 and LSM 980, the Multiplex 4Y mode acquires four image pixels in parallel. **d**, Available on LSM 980, the Multiplex 8Y mode acquires eight image pixels in parallel.

The Multiplex mode for Airyscan 2 is available on both LSM 900 and LSM 980, offering the ability to generate confocal (CO) or super-resolution (SR) images. The CO-2Y/SR-2Y and SR-4Y acquisition strategies are available on both LSM 900 (2Y and 4Y) and LSM 980 (4Y), providing parallelization factors of either 2× or 4× while keeping a 4× improvement in SNR (compared with that of traditional confocal microscopy) with a choice of confocal resolution or super-resolution (Table 1). The SR-4Y acquisition strategy provides simultaneous improvements in resolution, SNR, and imaging speeds to enable larger fields of view (FOVs) and/or higher volumetric imaging rates with better image quality than that of traditional confocal microscopy (Fig. 3). By varying the amount of parallelization and achievable resolution, the Multiplex mode provides researchers with the flexibility to match experimental needs. For maximum volume-acquisition rates, the Multiplex mode for Airyscan 2 on LSM 980 needs to be used.

Like the Fast mode for Airyscan on LSM 880, Multiplex mode for Airyscan 2 on LSM 980 utilizes an excitation shaping approach in combination with pinhole-plane imaging[1–5]. By slightly stretching the excitation beam along the *y*-dimension, the detection sampling approach of the Multiplex mode for Airyscan 2 provides an 8× parallelization in acquisition. Hence, the CO-8Y/SR-8Y uses very coarse acquisition stepping of the excitation laser, providing an 8× parallelization for increased imaging speeds (compared with those of traditional confocal microscopes) while also maintaining a 4× increase in SNR with a choice of confocal resolution or super-resolution. The increase in acquisition speed can be leveraged in single planes for ultrafast time series, rapid tiling of large areas, or fast volumetric time lapses. An example of the potential to increase the FOV with Multiplex mode, while retaining the temporal resolution, is shown in Fig. 3. For an additional demonstration of this improvement, we direct readers to a live-imaging example of motile cilia of brain ependyma imaged at 143 frames per second in Airyscan Multiplex CO-8Y mode, available at https://zeiss.wistia.com/medias/y849cr1oq9. This acquisition combines image quality and speed for detailed analysis of the ciliary beating direction and frequency. Additionally, Multiplex was leveraged to study chromosome movement during meiosis in starfish oocytes. Volumes of roughly 70 × 70 × 27 µm were acquired at diffraction-limited resolution every 2.4 s for 15 min. The acquired movie (available at https://zeiss.wistia.com/medias/p59hxxx9n7) shows the transport of chromosomes, labeled by histone 1–Alexa Fluor 568, in a starfish oocyte undergoing meiosis. It also reveals that concomitant with chromosome transport, the nucleolus (the large spherical structure) is disassembling.

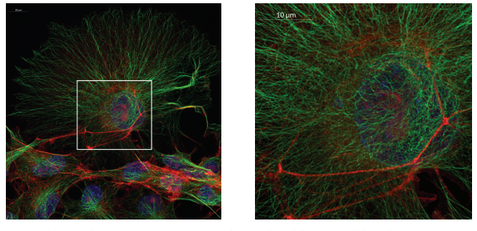

Figure 3 | Imaging at super-resolution with Airyscan Multiplex. Comparison of the FOVs a user can image at super-resolution in a given amount of time with the Airyscan SR (right) and Multiplex mode SR-4Y (left). The images shown are of COS-7 cells with labeled microtubules (α-tubulin 488; green) and actin (phalloidin 647; red).

Summary

With a focus on streamlined data-handling and decreased image-reconstruction times, the evolution of Airyscan 2 provides new data-handling concepts that yield 6.6× smaller data sizes and 5× faster image-reconstruction times. Further, optimized real-time acquisition strategies used with the LSM 9 series enable faster scan speeds for Airyscan 2, thus allowing for improved data throughput. The new and innovative imaging strategy of the Multiplex mode for Airyscan 2 allows scientists to address current developments in biomedical research in order to capture less signal and provide statistically sound data. With a focus on extending Airyscan imaging to larger model systems with lower expression levels, the Multiplex mode concept increases acquisition speeds while simultaneously providing super-resolution capabilities and a 4× improvement in SNR compared with that of traditional confocal imaging. The novel detection sampling concept allows for rapid volumetric imaging with unprecedented resolution beyond what is available in traditional confocal systems today.

Table 1 | Overview of the performance of Multiplex mode for Airyscan 2 on both LSM 900 and LSM 980 microscopes

LSM 900					
Mode	**Confocal**	**Airyscan SR**	**Multiplex SR-2Y**	**Multiplex SR-4Y**	**Multiplex CO-2Y**
Parallelization	1	1	2	4	2
Max resolution *x*/*y* (nm)	NA	120/120	140/140	140/140	180/180
Max resolution *z* (nm)	NA	350	450	450	550
Max. FPS @ 512 x 512	4.7	4.0	8.4	18.9	8.3
FPS @ max. FOV (mm)	0.5(@ zoom 0.5/SF18)	0.4(@ zoom 1.3/SF7)	0.8(@ zoom 1.3/SF7)	3.5 (@ zoom 1.3/SF7)	3.5 (@ zoom 1.3/SF7)
Processing 1k x 1k/150 slices	NA	<30 s	<30 s	<30 s	<30 s
SNR vs. conventional confoc.	NA	4–8x	4–8x + speed	4–8x + speed	NA
LSM 980					
Mode	**Confocal**	**Airyscan SR**	**Multiplex SR-4Y**	**Multiplex SR-8Y**	**Multiplex CO-8Y**
Parallelization	1	1	4	8	8
Max resolution *x*/*y* (nm)	NA	120/120	140/140	120/160	180/220
Max resolution *z* (nm)	NA	350	450	450	550
Max. FPS @ 512 x 512	6.1	4.7	25.0	47.5	34.4
FPS @ max. FOV (mm)	0.4 (@ zoom 0.6/SF20)	0.2 (@ zoom 1.7/SF7)	1.0 (@ zoom 1/SF12)	2.0 (@ zoom 1/SF12)	9.6 (@ zoom 1/SF12)
Processing 1k x 1k/150 slices	NA	<30 s	<30 s	<30 s	<30 s
SNR vs. conventional confoc.	NA	4–8x	4–8x + speed	4x + speed	NA

All values determined for a 63×/1.4-NA objective and a laser wavelength of 488 nm. The maximum x/y resolution was measured with nanoruler samples for SR modes. Maximal speeds were measured at variable zooms required for preferred sampling with selected regions of interest (e.g., the maximum number of frames per second (FPS) for confocal (confoc.) is sampled for super-resolution or diffraction-limited resolution). Hence, regardless of sampling requirements, the maximum achievable speed of the scanners at 512 × 512 is unchanged in confocal mode for both LSM 900 and LSM 980 (8 FPS and 13 FPS, respectively).

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